THE UNIVERSITY *of* EDINBURGH

EVALUATION OF PREDATORS AS SENTINELS FOR EMERGING INFECTIOUS DISEASES

ANNA LOUISE MEREDITH

PhD

THE UNIVERSITY OF EDINBURGH

2011

EVALUATION OF PREDATORS AS SENTINELS FOR EMERGING INFECTIOUS DISEASES

Declaration

I declare that this thesis has been composed by myself, that the work is my own, and that it has not been submitted for any other degree or professional qualification

…….

Anna Louise Meredith

ABSTRACT

New and emerging diseases in human and animal populations appear to be predominately associated with generalist pathogens that are able to infect multiple hosts. Carnivores are susceptible to a wide range of these pathogens and can act as effective samplers of their vertebrate prey, which are important reservoirs of many emerging diseases.

This thesis evaluates the utility of carnivores as sentinels for pathogens present in their prey by exploration of four selected pathogen-prey-sentinel combinations in three rural study sites of varying habitat in northern England and Scotland over a twenty-two month period (2007-2009). Selected pathogens were *Coxiella burnetii*, *Leptospira* spp., *Encephalitozoon cuniculi,* and rabbit haemorrhagic disease virus (RHDV*)*, selected prey species were wild rodents and rabbits, and selected carnivores were foxes, domestic cats and corvids. Seroprevalence to *C.burnetii*, *Leptospira* spp and *E.cuniculi* was assessed using adapted or novel test methodologies to enable their use for multiple mammalian species, however these were not applicable to corvids. RHDV seroprevalence was not assessed due to low acquisition of rabbit samples.

Overall, seroprevalence to all three pathogens was significantly higher in predators than prey, at 24.2% and 12.4 % for *C.burnetii*, 22.73% and 1.95% for *Leptospira* spp and 39.06% and 5.31% for *E.cuniculi* in predator and prey species respectively. A similar pattern was found in all study areas and was consistent irrespective of individual prey or predator species, although serological evidence of exposure to *E.cuniculi* was not detected in domestic cats in any area. A semi-quantitative assessment of the time and financial costs of the study approach and application to hypothetical examples indicates that sampling carnivores is a much more cost-effective approach to pathogen detection than sampling prey.

The results indicate that carnivores can act as useful sentinels for broad-scale detection of pathogen presence and relative levels of prevalence in prey and predator populations. Careful selection of predator species and methods of sample acquisition are necessary to maximise their utility, and issues associated with diagnostic test performance and validation must also be acknowledged. Suggestions are made as to how this principle might be applied to future surveillance programmes. In addition, the study is the first report on the seroprevalence of *C.burnetii, Leptospira* spp and *E.cuniculi* in multiple wildlife species (field voles, bank voles, wood mice, foxes), the first detection of antibodies to *C. burnetii* in wildlife and cats, the first detection of antibodies to *L mini, L hardjo prajitno* and *L hardjo bovis* in wild rodents, and to *L mini* in cats, and the first detection of antibodies to *E.cuniculi* in wild rodents and foxes in the UK.

Acknowledgements

Heartfelt thanks to Darren Shaw for all the encouragement, support, supervision and patience, to Sarah Cleaveland for the original inspiration behind this thesis and her continued input and support, and to Jeremy Brown for laboratory support and supervision. Thanks to all the other colleagues who helped me, in particular Alan Pemberton, Lorna Hume, Jennifer Harris, Edith Paxton and Jo Halliday.

Thanks to United Utilities, Robert Balfour, and Forestry Commission for access to sites, to Alex Moir, Jim Cowe, Leon and all those who collected foxes, and to Laurie and Sue Walton for trapping assistance and their warm hospitality.

Thanks to Defra for funding support

And to AH, AH and SH for their love, understanding, patience, and support.

Table of contents

ABSTRACT 3
Acknowledgements 4
Table of contents 5
List of Figures 8
List of Tables 11

Chapter 1: The use of animals as sentinels for disease surveillance 13
1.1 Introduction 13
1.1.1 Emerging and re-emerging infectious disease 14
1.1.2 What drives disease emergence? 17
1.1.3 Surveillance of emerging infectious diseases 30
1.1.4 Carnivores/predators as sentinels 43
1.2 Research objectives 49

Chapter 2: Selection of pathogen-prey-predator combinations 51
2.1 Introduction 51
2.2.1 Bacterial pathogens 57
2.2.2 Protozoal, microsporidial and fungal pathogens 60
2.2.3 Viral pathogens 64
2.4 Selection of predator and scavenger species 67
2.5 Summary of selected combinations 71

Chapter 3. Study site selection and prey and predator sample collection methods 72
3.1 Selection of study sites 72
3.2 Licensing 77
3.3 Sample collection 77
3.3.1 Wild rodents 77
3.3.2 Rabbits and Rabbit Haemorrhagic Disease Virus (RHDV) 83
3.3.3 Foxes 83
3.3.4 Cats 84
3.3.5 Corvids 84
3.4 Results 85
3.4.1 Prey species 85
3.4.2 Predator species 92
3.4.3 Sampling timeline of prey and predators 96
3.4.4 Age and sex distribution of predators 97
3.4.5 Samples submitted for serological testing 98
3.5 Discussion 99

Chapter 4. *Coxiella burnetii* 110
4.1 Introduction 110
4.2 Test options for *C. burnetii* 113
4.2.1 Indirect immunofluorescence assay (IFA) 115
4.2.2 Complement fixation test (CFT) 116
4.2.3 Enzyme-linked immunosorbent assay (ELISA) 117
4.3 Test selection and modification 117

4.3.1 Materials and methods 119
4.4 Results 121
4.4.1 Raw S/P values 122
4.4.2 Thresholds 126
4.4.3 Seroprevalence 131
4.5 Discussion 134

Chapter 5. *Leptospira* spp. 139
5.1 Introduction 139
5.2 Test options for *Leptospira* spp. 140
5.2.1 Microscopic agglutination test (MAT) 142
5.2.2. ELISA 144
5.3 Materials and methods 147
5.3.1 Development of an indirect ELISA for leptospiral antibodies 147
5.3.2 MAT 154
5.3.3 Data analysis 155
5.4 Results 156
5.4.1 Raw S/P values 157
5.4.2 Thresholds 160
5.4.3 Seroprevalence 165
5.4.4 Comparison with MAT results 168
5. 5 Discussion 172

Chapter 6. *Encephalitozoon cuniculi* 179
6.1 Introduction 179
6.2 Test options for *E.cuniculi* 182
6.2.1 ELISA and IFA 182
6.2.2 Direct agglutination test 182
6.3 Materials and methods 183
6.3.1 Direct agglutination test development 183
6.4 Results 188
6.4.1. Seroprevalence 189
6.4.2 Patterns of seroprevalence 191
6.5 Discussion 192

Chapter 7. Effectiveness of the study approach in demonstrating the proof of principle and addressing the key research questions 196
7.1 Introduction 196
7.2 Summary of animal sampling, testing, seroprevalence and co-infection 197
7.3 Relationships between predator and prey seroprevalence 201
7.4 Cost-effectiveness of the study approach 221
7.4.1 Acquisition and costs of prey samples 223
7.4.2 Acquisition, quality and costs of predator samples 227
7.4.3 Test methodology and development 231
7.5 Test performance and validation 236
7.6 Conclusions 239

Chapter 8. How could predator sampling be incorporated into future surveillance programmes for endemic pathogens or those representing higher level threats? 241
8.1 Introduction 241
8.2 Means of incorporation of predators as sentinels into future surveillance programmes 244

References 254
APPENDIX 1 292
APPENDIX 3 293
APPENDIX 5.1 295
APPENDIX 5.2 299
APPENDIX 6 302

List of Figures

Figure 1.1: Key components and attributes of the sentinel framework (from Halliday et al 2007) 36
Figure 1.2: The sentinel framework in context (from Halliday *et al* 2007) 40
Figure 1.3. Relative seroprevalence of infections that can be transmitted by consumption of infected material 46

Figure 3.1 Map of Northern England and Scotland indicating initial study sites considered for the study (with filled boxes indicating sites chosen (see below)). 73
Figure 3.2 General map of borders area a) Ordnance Survey b) Satellite image..... 75
Figure 3.3 General map of Cumbria area a) Ordnance Survey b) Satellite image.... 75
Figure 3.4 General map of Pentlands area a) Ordnance Survey b) Satellite image ... 76
Figure 3.5 Examples of variety of habitat for wild rodent trapping: (a) rough pasture grazed intermittently by sheep (Cumbria); (b) clear cut area in cultivated conifer forestry (Borders) (c) deciduous woodland strip adjacent to grazed fields (Pentlands) 78
Figure 3.6 Visual evidence of rodent activity (field vole) demonstrated by the presence of droppings (a) and grass clippings (b). For field voles presence of *Juncus* spp. (c) or *Deschampsia* spp. (d) was frequently indicative of the likely presence of runs as these are favoured food items. 78
Figure 3.7. Ugglan trap with storm cover 79
Figure 3.8 Schematic diagram of trapping grid 79
Figure 3.9 Prebaited Ugglan trap (a) and in situ (b) 80
Figure 3.10 Map of a typical study site with schematic representation of a trapping grid in place 80
Figure 3.11 Once trapped, rodents were transferred without handling into a plastic bag. 82
Figure 3.12 Maps of the three study sites indicating trapping grid locations and pie charts giving total numbers of rodents caught by species 86
Figure 3.13 Barplot of the numbers of rodent prey species trapped per area and season 87
Figure 3.14 Seasonal variations in numbers of prey species collected from each study area: a) Wood mice b) Bank vole c) Field vole. 89
Figure 3.15 Boxplot of overall trapping success (number of rodent captures per 100 trap nights) per season for each study area. Horizontal line indicates median value, upper whisker extends to the maximum data point within 1.5 box heights from the top of the box, lower whisker extends to minimum data point within 1.5 times box heights from the bottom of the box. 90
Figure 3.16 Numbers of cats and foxes sampled from each study area 92
Figure 3.17. Maps indicating locations and numbers of predators (foxes and cats) in relation to rodent trapping sites for a) Borders, b) Cumbria and c) Pentlands study areas. Fox home ranges are estimated at 10km^2 93
Figure 3.18 Timeline of when prey and predator species were sampled during the study period from January 2007 to December 2009. Trapping periods for prey species are indicated (seasons 1-4). 97
Figure 3.19 Box plot of age and sex of cats in each study area (as for Figure 3.15) . 97
Figure 3.20 Age category and sex distribution of foxes sampled (n=126) 98

Figure 4.1 Probability plots for residuals for a) raw S/P and b) log10 S/P values for the *C. burnetii* ELISA test. 121
Figure 4.2 Plots of S/P values for each species per site. Horizontal bar represents the median value. These dot plots have been jigged so that width of the "blob" of overlying dots reflects, but is not an exact representation of, the numbers of animals with this S/P value. 123
Figure 4.3 Relationship between median S/P values in predators and prey 125
Figure 4.4 Proportions of predator and prey species above differing S/P thresholds in each study area. (Pentlands Exc Site refers to the Pentlands site with one study site accounting for 63% of all S/P values > 15% excluded) 126
Figure 4.5 Proportion of each individual species above differing thresholds in each study area (BV = bank vole, FV = field vole, WM = wood mouse). (Pentlands Exc Site refers to the Pentlands site with one study site accounting for 63% of all S/P values > 15% excluded) 127
Figure 4.6 a) Distribution of S/P values for prey species. Dotted line at 15% indicates the selected threshold that appears to separate two populations 129
Figure 4.6b) Distribution of S/P values for predator species. Dotted line at 25% indicates the selected threshold that appears to separate two populations 130
Figure 4.7 Relationship between seroprevalence in predators and prey in the three study areas 133
Figure 4.8 Seroprevalence by season in foxes and prey in the Pentlands area 133

Figure 5.1 Plate template for serial dilutions of reference leptospiral antisera. All wells were coated with mixed leptospiral extract. 149
Figure 5.2 Optical densities of 11 reference leptospiral antiserum at dilutions of 1:10, 1:100, 1:1000, 1:10,000 tested by ELISA against the extracted leptospiral antigen mix. 150
Figure 5.3 Plot of optical density of reference leptospiral antisera 1,2,3,4,7,8,9,11,12,14, and 15 to individual extracts of leptospira cultures 1 to 14. The diameter of the symbol is proportionate to the optical density of the ELISA test result. 151
Figure 5.4 Optical densities obtained by ELISA of 1:500 dilutions of reference antisera against four different concentrations of mixed leptospiral antigen solution. 152
Figure 5.5 Optical densities obtained by ELISA of serial dilutions of reference antisera 7 and 12 with antisera 2 as control, against 0.1 µg/ml leptospiral antigen mix. 153
Figure 5.6 Probability plots for residuals for (a) raw S/P values and (b) log 10 S/P values 155
Figure 5.7 Dot plots of raw S/P values by species and study area. Horizontal bar indicates the median value for each species in each study area. These dot plots have been jigged so that width of the "blob" of overlying dots reflects, but is not an exact representation of, the numbers of animals with this S/P value. 157
Figure 5.8 Proportions of predator and prey species above increasing S/P thresholds up to 50% in each study area 160
Figure 5.9 Proportion of each individual species above increasing S/P thresholds in each study area (BV = bank vole, FV = field vole, WM = wood mouse). 161
Figure 5.10 Distribution of S/P values for prey species. Dotted line at 10% indicates

the selected threshold that appears to separate two populations 163
Figure 5.11 Distribution of S/P values for predator species. Dotted line at 60% for cats and 30% for foxes indicates the selected threshold that appears to separate two populations 164
Figure 5.12 Relationship between seroprevalence in predators and prey in the three study areas 166
Figure 5.13 Seroprevalence of foxes and prey in the Pentlands area by season, indicating a possible lagged effect between seasons 3 and 4 in Pentlands 167
Figure 5.14 ROC curves for the ELISA test as compared to the MAT for *Leptospira spp.* (red line = sensitivity, blue line = specificity; dotted lines = species test threshold)..... 170

Figure 6.1 Reducing gel of DAT positive sera. Rabbit = E.cuniculi positive control serum. UF=unfiltered serum, F=filtered serum, R= recovered proteins from filter. 186
Figure 6.2 Direct agglutination test plate for *E.cuniculi* antibodies in a) rodent serum samples (positive control 1AB, negative control 1CD, positive sera 4GH, 5AB, 9EF and b) fox serum samples (positive control 1AB negative control 1CD, numerous positive sera)..... 187
Figure 6.3 Seroprevalence in fox and prey in each study area..... 191
Figure 6.4 Seroprevalence in foxes and prey by season, a) for the study overall, b) by season 191

Figure 7.1 Numbers of co-infections in animals tested for all three pathogens (n=817) 200
Figure 7.2 Relationships between seroprevalence for all three pathogens in each study area..... 204
Figure 7.3 (repeated from 3. 17a) Location maps of cats and foxes in relation to trapping grids and fox home ranges (estimated at 10km^2) in the Borders, as an example of the home ranges and different degrees of overlap between prey, foxes and cats 205
Figure 7.4 Theoretical sample size of predators and prey required for absence of disease (95% confidence) at observed seroprevalence levels for each pathogen in each study area, assuming a prey density of 1000/km2, predator density of 2/ km^2 and a study area of 100km^2 219

Figure 8.1 A framework for predator as sentinels (adapted from Halliday et al 2007) 243

List of Tables

Table 1.1: Summary of applications of animal sentinels for environmental and infectious hazards. (From Halliday *et al*, 2007) 33
Table 1.2: Important animal host categories for zoonotic diseases (from Cleaveland *et al.,* 2001) 45

Table 2.1 Pathogens considered for investigation in this study but rejected 56
Table 2.2: Summary table of selected pathogen-prey-sentinel frameworks 71

Table 3.1 Summary of trapping seasons for wild rodents 81
Table 3.2 Summary of prey and predator species collected from the three study sites 85
Table 3.3 Summary of prey species trapped per season and area 87
Table 3.4 Trapping success (captures per 100 trap nights (tn)) for each study area per season. 90
Table 3.5 Trapping success (captures per 100 trap nights (tn)) for each rodent species per study area and season. 91
Table 3.6 Age and sex of prey species caught per area 91
Table 3.7 Numbers and species of serum samples tested for *C. burnetii*, *Leptospira* spp and *E.cuniculi* from each study area 99

Table 4.1 Numbers of serum samples tested for *C. burnetii* by area, predator or prey status, species and season 122
Table 4.2 Median S/P values (with ranges) and mean (with SE mean) values for *C. burnetii* ELISA test results in prey and predator species. 124
Table 4.3 Seroprevalence for *C. burnetii* (with 95% confidence intervals) for predators and prey overall 131
Table 4.4 Seroprevalence for *C. burnetii* (with 95% confidence intervals) for prey and predator species in each study area 131

Table 5.1 Reference type cultures of *Leptospira* maintained by live culture and used as the basis for the ELISA 148
Table 5.2 Numbers of serum samples tested for *Leptospira spp*. by area, species, predator or prey status and season 156
Table 5.3 Median S/P % (with ranges) and mean (with SE mean) values for *Leptospira spp*. antibodies by ELISA testing in prey and predator species overall by study area 158
Table 5.4 Median S/P % (with ranges) and mean (with SE mean) values for *Leptospira spp*. antibodies by ELISA testing in individual species 159
Table 5.5. Seroprevalence for *Leptospira* spp. (with 95% confidence intervals) for the study overall. 165
Table 5.6 Seroprevalence *for Leptospira* spp. (with 95% confidence intervals) in each species by study area. 165
Table 5.7 Seroprevalence for *Leptospira spp*. using the MAT 168
Table 5.8 Observed frequencies of test results for ELISA compared to MAT 169
Table 5.9 ELISA test sensitivities and specificities (with 95% confidence intervals) for each species when compared to the MAT 169

Table 5.10 Number of animals of each species giving a positive result to the *Leptospira* serovar pools 1-6. (29 animals were positive to more than one pool). 171
Table 5.11 MAT results for individual serovars in each species (46 samples)..... 172

Table 6.1. Species tested for *E.cuniculi* antibodies in each study area and season . 189
Table 6.2 Seroprevalence for *E.cuniculi* in predators and prey for the study overall 189
Table 6.3 Seroprevalence to *E.cuniculi* in predator and prey species in each study area 190

Table 7.1 Seroprevalence for the study overall for *C.burnetii*, *Leptospira* spp. and *E. cuniculi* (confidence intervals not shown)..... 196
Table 7.2 Seroprevalence in each study area for *C.burnetii*, *Leptospira* spp. and *E. cuniculi* (confidence intervals not shown 199
Table 7.3 Sample size calculations for absence of disease (95% and 99% confidence) assuming a prey density of 1000/ km^2 , predator density of 2/ km^2 and study area of 100 km2. (* = predator density too low to declare absence of disease at this seroprevalence, as in effect would require sampling of > 2 predators/km^2). 218
Table 7.4 Assessment of trapping efficiency in each study area 225
Table 7.5 Person hours and estimated costs of rodent trapping..... 226
Table 7.6 Advantages and disadvantages of the test methodologies used..... 234

Table 8.1 Other candidate pathogens suitable for investigation (repeat of Table 2.1) 250

Chapter 1: The use of animals as sentinels for disease surveillance

1.1 Introduction

Emerging infectious diseases of humans, domestic and wild animals can have a significant impact on human and animal health and welfare, global trade and economies, and biodiversity (Binder et al., 1999; Daszak et al., 2000; Morens et al., 2004; Woolhouse, 2002a). In order to minimise the impact of emerging infectious diseases, there is a fundamental need for appropriate disease surveillance. Disease surveillance is defined by the World Health Organisation (WHO) as the process of ongoing systematic collection, collation, analysis and interpretation of data and the dissemination of information to those who need to know in order for action to be taken (World Health Organisation, 2001). It refers to the continuing scrutiny of all aspects of occurrence and spread of diseases that are pertinent to effective control (Last, 2001). In addition to accurately detecting the presence or absence of potential pathogens, effective surveillance aims to inform policy and response to emerging disease situations. Animals are thought to be the source of more than 70% of all emerging infections in humans (Taylor et al., 2001; Woolhouse and Gowtage-Sequeria, 2005) and thus pathogen surveillance in both domestic and wild animals is essential to detection, intervention and control of emerging infectious diseases at a regional, national and global level (Kuiken et al., 2005; Merianos, 2007).

The aim of this study is to explore the use of one particular group of animals, carnivores or predators, in disease surveillance, by investigating how they might reflect the diseases present in their prey populations, and potentially act as sentinels for disease in these populations (see 1.2). This approach may be useful for investigating a pathogen or disease of significance or interest in the prey population itself, or as a method for monitoring prey populations as potential reservoirs of pathogens that may infect other animals or humans. The hypothesis is that the use of predators as sentinels may provide a more efficient and cost-effective means of disease surveillance than by targeting their prey species directly. If they can fulfil this role of sentinel, predators will provide a means of surveillance for diseases in their prey populations, including those that are of potential concern to other animal

or human populations, and those that are emerging. This surveillance tool could then be considered by relevant human and animal health organisations for use in disease monitoring and control programmes.

1.1.1 Emerging and re-emerging infectious disease

Emerging infectious diseases (EIDs) have been defined as "infections that have newly appeared in a population or have existed previously but are rapidly increasing in incidence or geographic range" (Morse, 1995). The World Organisation for Animal Health, or Office International des Epizooties (OIE) gives a more detailed definition and refers to an EID as " a new infection resulting from the evolution or change of an existing pathogenic agent, a known infection spreading to a new geographic area or population, or a previously unrecognized pathogenic agent or disease diagnosed for the first time and which has a significant impact on animal or public health" (World Organisation for Animal Health, 2010).

One issue when looking at the field of emerging diseases is that the terms "new", "emerging" or "emergent" and "re-emerging" are widely used in the scientific literature in relation to infectious diseases but are often not clearly defined, and interpretation is assumed. In general, definitions of "emerging" or "re-emerging" are intended to reflect differences in epidemiology that distinguish long-term global trends in incidence (emerging) from short-term or local increases in incidence (re-emerging). The term "new" should, in principle, be clear, referring to a disease that is novel and not previously described. However the above definitions of EIDs (Morse, OIE) refer to them as "new", "previously unrecognised or "newly appeared" which blurs the distinction between new and emerging, and the two terms are frequently used synonymously. An emerging disease can be "new" but a "new" disease may not necessarily be "emerging", according to the OIE definition, unless it has a significant impact on animal or public health. Existing diseases may be highly significant and have a major impact on human or animal health but may not be emerging in many parts of the world, such as malaria (World Health Organisation, 2009) and rabies (Coleman et al., 2004). An existing disease may be perceived to be "emerging" when attention and focus are drawn to it, so that awareness, surveillance

and reporting are increased. Whether or not, or to what extent, a disease is truly new or emerging can be in question, as it may well be that it has not been looked for or described before, but, once recognised and its potential origin investigated, evidence comes to light that reveals the pathogen may have been present for some time; for example, chytridiomycosis has been traced back to *Xenopus laevis* in Africa in 1938 (Weldon et al., 2004), and rabbit haemorrhagic disease virus, which emerged as a lethal new disease in China in 1984, has been found to have been circulating in an avirulent form in wild rabbits in the UK for at least 50 years (Moss et al., 2002).

In addition to the three types of EID listed above in the OIE definition, some definitions include as examples of EIDs old or known infections that are re-emerging or re-appearing after a significant decline in incidence, as a result of antimicrobial resistance or breakdowns in public health measures, (e.g. the journal Emerging Infectious Disease (http://www.cdc.gov/ncidod/EID/index.htm), NIAID). A re-emerging pathogen has been defined as "one whose incidence is increasing in an existing host population as a result of long-term changes in its underlying epidemiology" (Woolhouse, 2002). Whilst it could be assumed that re-emergence therefore occurs in the same geographical areas and population in which the infections once existed or have been existing at a low level (endemic), the term is also used synonymously with emergence, for example to describe the recent spread of Bluetongue virus into many parts of Europe and the south-eastern USA (MacLachlan and Guthrie, 2010) where it has not occurred before. Examples of human re-emerging diseases in some parts of the world are multi-drug resistant tuberculosis (Espinal, 2003) and drug-resistant malaria (Morens et al., 2004).

Although it would be desirable to have a tight definition of EID that the scientific community stays adherent to, due to the potential confusion over terminology this thesis will define EID in its broadest sense as discussed above to refer to:

- New infections resulting from changes or evolution of existing pathogens (e.g. HIV/AIDS, Nipah virus, Hendra virus, swine-origin influenza)
- Known existing infections spreading to new geographic areas or populations (e.g. monkeypoxvirus, dengue fever, Lyme borreliosis, bluetongue virus)

- Previously unrecognized infections appearing in areas undergoing ecologic transformation that *may* have a significant impact on human or animal health (e.g. chytridiomycosis in amphibians, white nose syndrome in bats, *Cryptococcus gattii* in humans)
- Old, previously recognised infections that are re-emerging as a result of antimicrobial resistance in known pathogens, or breakdowns in public or animal health measures (e.g. measles, multi drug resistant tuberculosis, MRSA).

EIDs that affect humans and animals impact on human society, animal production and welfare, and conservation of biodiversity (Daszak et al., 2000). They present challenges in terms of protection of public health, and the economic costs associated with trade and travel restrictions, loss of food production, and vaccination or treatment regimes. As examples of the impact of human and animal diseases:

- The global AIDS pandemic currently affects 33 million people and in 2008 cost $7.7 billion in international assistance (UNAIDS, 2008).
- Estimations by the World Bank are that the 2003 severe acute respiratory syndrome (SARS) epidemic was responsible for a 2% fall in gross domestic product (GDP) across East Asia, even though it killed only 774 people (WHO, 2003), and estimate the loss of $800 billion in GDP world-wide if an avian influenza pandemic strikes (Brahmbhatt, 2005).
- The 2001 foot and mouth disease outbreak in the UK led to the slaughter of 2,382,000 sheep and cattle (Defra, 2010)
- Bovine spongiform encephalopathy (BSE) lost the UK approximately £607 million annually (Pretty et al., 2000) at the height of the disease outbreak in 1996/7.

Effects on wildlife can also be significant, for example canine distemper virus has been implicated in extinctions of populations of African wild dogs and black-footed ferrets (Ginsberg et al., 1995; Williams et al., 1988), and the amphibian fungal disease chytridiomycosis has caused mass mortalities, population declines and possible global extinctions of endangered species of frog (Rosenblum et al., 2010).

Over the last 30 years interest in the concept of emerging infectious diseases has developed into an entire discipline that continues to gain prominence (Brown, 2004), and there are a number of surveys and reviews that analyse emerging disease events and the factors associated with them in an attempt to improve understanding of how and why pathogens emerge (e.g. (Blancou et al., 2005; Brown, 2004; Cleaveland et al., 2001; Cleaveland et al., 2007; Greger, 2007; Jones et al., 2008; McMichael, 2004; Taylor et al., 2001; Woolhouse et al., 2001; Woolhouse, 2002; Woolhouse and Gowtage-Sequeria, 2005). New diseases are currently being detected at a rate of approximately one per year (Woolhouse, 2002). Between 1940 and 2004, 335 emerging infectious disease events have been reported globally in the human population (Jones et al., 2008), with an increasing incidence over this period, and a peak in the 1980s. In their analysis of these human EID events Jones *et al* suggest that this is probably a real increase, not just increased reporting effort, which would support suggestions that the threat of EIDs to global health is indeed becoming greater (Institute of Medicine, 1992). However, Jones *et al* (2008) base reporting effort only on one English – language journal (the Journal of Infectious Disease) so this may not accurately reflect the true situation.

1.1.2 What drives disease emergence?

For an infectious disease epidemic to persist a minimum number, or threshold level, of susceptible hosts is required. This minimum host population size and density required to allow a pathogen to persist is referred to as the critical community size (Anderson R.M. and May, 1992b; Kermack and McKendrick A.G, 1927). The expected number of secondary cases resulting from a single infection in a susceptible population is referred to as the basic reproduction number, R_0 ; thus a disease can emerge and persist if R_0 is >1, but will ultimately die out if R_0 <1 (Anderson R.M. and May, 1992a). Persistence therefore depends on there being sufficient susceptible hosts to infect. In a population of fixed size the number of susceptible hosts will decrease, as a disease spreads, as more infected individuals die or become immune. However, R_0 is not a fundamental fixed characteristic of a pathogen but is a characteristic of a particular pathogen population in a particular host population at a specific time, and will vary depending on the host population density, its behaviour

and ecology, and how these affect contact rates (Dietz, 1993).

In reality human or animal populations are rarely closed and fixed in size due to immigration and birth, which will bring in new susceptible hosts. Variation in the number of susceptible hosts and other extrinsic factors, such as the institution of control measures, will change the observed reproduction ratio or number to R_t, the average number of secondary cases arising from a single case infected at time *t* (Haydon et al., 2003). In most cases $R_t < R_0$ and if $R_t > 1$ the epidemic is growing or emerging, and if <1 the epidemic is in decline although this does not necessarily mean that $R_0 < 1$. Estimation of the reproduction ratio is thus important for understanding the dynamics of EIDs and for evaluating the impact of control measures that are applied.

The major factors that influence and drive disease emergence include a zoonotic origin of the pathogen, its taxonomy and host range, anthropogenic factors and the routes of transmission.

Zoonotic origins of pathogens and wildlife reservoirs

The close and important links between the infectious diseases of humans, their domestic animals, and wild animals are well recognised (Cleaveland et al., 2001), with 58% (816) of the 1,407 recognised species of human pathogen classified as zoonotic (Taylor et al., 2001). Zoonoses are defined as " diseases and infections that are naturally transmitted between vertebrate hosts and man" (World Health Organisation, 1959) and have been identified as representing 73% of the 177 currently recognised emerging and remerging diseases (Woolhouse and Gowtage-Sequeria, 2005). Zoonoses can be classified in many ways, such as by organ system affected, animal species of origin, and mode of transmission, or according to their likely evolutionary history (Bennett and Begon, 1997; Hart et al., 1999). Using the latter classification human specific infections with a temporally distant non-human source, but which now have adapted to mainly or exclusively human to human transmission such as measles and the common cold, can be considered "old zoonoses". "Recent zoonoses" refer to diseases such as HIV/AIDS ,shown to have a

primate source (Gao et al., 1999; Hirsch et al., 1989) and SARS, shown to have bats as the natural host reservoir, with civets acting as an intermediate host (Lau et al., 2010; Li et al., 2005; Song et al., 2005) that have now adapted to human to human transmission. Infectious diseases with an animal reservoir that are occasionally transmitted to humans, such as rabies, monkeypox, Q fever and brucellosis can be classified as "established zoonoses" (Hart et al., 1999), while "new and emerging zoonoses" describe those diseases with an animal reservoir which have only recently been observed to spread to humans, such as rodent-borne Hantaviruses (Klein and Calisher, 2007), bat henipaviruses, Nipah and Hendra (Wild, 2009) and Ebola virus (Groseth et al., 2007). These pathogens rely on continued re-introduction into human populations from their animal reservoirs. The distinction between "recent" and "new" is somewhat arbitrary, and will change as time progresses – a new disease may be classified as recent within a few years so these terms can be confusing.

Bennett and Begon (1997) were the first to use the term "parazoonoses" to describe infectious diseases that are endemic or epidemic in humans that change in virulence due to an input of genes from non-human pathogens(Bennett and Begon, 1997). A very recent example is that of the 2009 swine-origin influenza A (H1N1) pandemic (Malik Peiris et al., 2009; Neumann et al., 2009), which is believed is likely to have arisen through re-assortment of two or more viruses of swine origin. H1N1, H1N2 and H3N2 swine influenza viruses have occasionally infected humans in the past but in the 2009 pandemic an initial zoonotic event then led to sustained human to human transmission (Malik Peiris et al., 2009).

The process by which a pathogen undergoes evolutionary transformation from an animal pathogen into a specialised pathogen of humans has been categorised into five stages (Wolfe et al., 2007):

- Stage 1 - a pathogen that is present in animals only;
- Stage 2 - an animal pathogen that has been transmitted from animals to humans (primary infection) but is not transmitted between humans (secondary infection) e.g. anthrax, tularaemia, rabies, West Nile virus;

- Stage 3 - an animal pathogen that can undergo only a few cycles of secondary transmission between humans e.g. Ebola, Marburg and monkeypox viruses;
- Stage 4 - a pathogen that has a natural cycle of infecting humans by primary transmission from an animal but also undergoes long cycles of secondary transmission between humans e.g. Chagas' disease, dengue fever, influenza A;
- Stage 5 - a pathogen exclusive to humans e.g. measles, mumps, smallpox.

Stage 5 pathogens could become confined to humans either through co-speciation of an ancestral pathogen present in the common ancestor of humans and chimpanzees when their lineages diverged approximately five million years ago, or else has colonised humans more recently and evolved into a specialised human pathogen. These stages have important implications for disease surveillance, as surveillance of a pathogen in animal populations (stages 2-4), or even a stage 1 pathogen that has been identified as having the potential to transform to stage 2, could potentially lead to interventions that could prevent a pathogen becoming a major human threat.

Wolfe et al (2007) looked at 25 human diseases that pose, or have posed in the past, the heaviest global burdens and found that there are notable differences between tropical diseases and temperate diseases in terms of their animal origins. Tropical diseases, such as AIDS, dengue fever, yellow fever, are more likely to have wild primate origins, whereas temperate diseases, such as measles, influenza A, rotavirus A, tetanus, tuberculosis, are more likely to have arisen from domestic animals.

Although there are still major gaps in our understanding of the origins of many human infectious diseases – for example smallpox and malaria (Wolfe et al., 2007) – regardless of exact origin it is apparent that there are two major mechanisms or patterns of zoonotic disease emergence (Bengis et al., 2004; Cleaveland et al., 2007). In the first, the pathogen has its origins as a zoonosis, and although transmission of a pathogen from animals to humans is a rare event once it has occurred, human-to-human transmission maintains the infection for some period of time or permanently,

(for example HIV/AIDS, influenza A, Ebola virus and SARS). The second pattern is characterised by direct or vector-mediated transmission from domestic or wild animals as the principle source of human infection. Pathogens such as rabies and other lyssaviruses, Nipah virus, West Nile virus, Hantavirus and the agents of Lyme borreliosis, plague, tularaemia, leptospirosis and ehrlichiosis follow this pattern.

The role of wildlife in emerging and re-emerging zoonoses is therefore widely recognised (Bengis et al., 2004; Chomel et al., 2007; Karesh et al., 2005; Kruse et al., 2004; Williams et al., 2002). Opportunities for contact between wildlife and humans are increasing due to human population expansion and encroachment on wildlife habitat, changes in agricultural practices, wildlife trade and translocation, consumption of bushmeat, and ecotourism (see anthropogenic factors below). Captive wild animals and exotic pets can also be a source of zoonoses, such as salmonellosis from pet reptiles, monkeypox from imported African rodents, and *Mycobacterium tuberculosis* from captive elephants (Bender and Shulman, 2004; Kile et al., 2005; Michalak et al., 1998). Global wildlife trade, for pets, food or use in traditional medicine, provides another means of contact and potential disease transmission between man and animals. For the exotic pet trade alone, an estimated 40,000 primates, 4 million birds, 640,000 reptiles and 350 million tropical fish are transported live internationally every year in an industry worth an estimated $6 billion (Check, 2004). Karesh *et al* (2005) estimate conservatively that tens of millions of wild animals are transported annually in East and Southeast Asia both regionally and from around the world, and in Central Africa an estimated 570 million wild animals are consumed annually as bushmeat. Globally, it is suggested that “at least some multiple of one billion direct and indirect contacts among wildlife, humans and domestic animals result from the wildlife trade annually” (Karesh et al., 2005).

The interactions between domestic and wild animal populations have led to the terms spillover and spillback being used in relation to infectious disease in wildlife. Which term is used depends on the population of primary interest, but usually spillover refers to transmission from domestic animals to wildlife, and spillback refers to the

subsequent transmission from wildlife back to domestic animals (Palmer, 2007; Rhyan and Spraker, 2010). Tuberculosis due to *Mycobacterium bovis* provides a good example of an important zoonotic disease that is re-emerging in many parts of the word due to wildlife reservoirs (Palmer, 2007) ; badgers (*Meles meles*), brushtail possums (*Trichosurus vulpecula*), White-tailed deer (*Odocoileus virginianus*), bison (*Bison bison*) and African buffalo (*Syncerus caffer*) are examples of wildlife that are maintenance hosts of *M. bovis,* i.e. the infection will persist. There is growing understanding that these species can be the main source of infection for both domestic animals and other protected wildlife species. The presence of these wildlife reservoirs is the direct result of spillover from domestic livestock, in combination with anthropogenic factors (see 1.1.2.5) such as wildlife translocation, supplemental feeding of wildlife and wildlife populations reaching densities beyond habitat carrying capacities. However, attempts to eradicate *M. bovis* from domestic livestock are impeded by spillback from wildlife reservoirs (Palmer, 2007).

Pathogen taxonomy

The type of pathogen (viruses (including prions), bacteria, fungi, protozoa or helminths) has an impact on its likelihood of becoming an EID, and viruses are the largest taxonomic group of pathogens associated with disease emergence in humans and domestic animals (Cleaveland et al., 2001; Woolhouse and Gaunt, 2007). Viruses are also more likely to be the cause of diseases listed by the OIE as being transboundary and of socioeconomic concern due to their potential to spread rapidly and cause high mortality (Cleaveland et al., 2001). Of the 177 human pathogens classified as emerging (Woolhouse and Gowtage-Sequeria, 2005), 37% are viruses, and 55% and 59% respectively of emerging pathogens of domestic livestock and carnivores are viruses (Cleaveland et al., 2001). For human pathogens, viruses are more than four times more likely to be emerging than other taxonomic groups of pathogens, i.e. the relative risk of emergence (the proportion of emerging divided by the proportion not emerging) for viruses is 4.34 (n=77), and is even higher for emerging pathogens of domestic livestock and carnivores (RR for emergence 5.6 (95% CI 2.81-11.43) and 11.07 (2.75-24.0) respectively) (Cleaveland et al., 2001). In particular, RNA viruses are disproportionately represented among pathogens that

have caused disease emergence by jumping from an animal host species to humans, both in the distant past ("old" zoonoses, e.g. measles, smallpox, common cold) and more recently ("recent" or "new and emerging" zoonoses, e.g. HIV/AIDS, H5N1and H1N1 influenza A, Hepatitis E and SARS coronavirus).

It is not only in domestic animals that EIDs are an issue. In a survey of emerging infectious pathogens of wildlife, it was also found that the majority (42%) of the 31 pathogens identified were viruses (Dobson and Foufopoulos, 2001), and typically involve a jump from one animal host species to another, for example canine distemper virus (transmitted from dogs to seals and lions).

The relative difficulty of treating and controlling the spread of viral diseases, their higher mutation rates, particularly of RNA viruses, short generation times, and ability to undergo recombination and genome segment re-assortment have been put forward as possible explanations for the dominance of viral pathogens as the agents of emerging diseases (Cleaveland et al., 2007; Domingo, 2010; Domingo and Holland, 1997; Graham and Baric, 2010).

Bacteria make up the second largest group of pathogens associated with disease emergence in both humans and domestic animals, followed by protozoa, helminths and fungi. Although viruses cause the largest proportion of emerging diseases, analysis by Jones et al (2008) reveals that bacterial (including rickettsial) diseases account for the majority (54.3%) of reported EID events globally between 1940 and 2004, and viruses only accounted for 25.4%. These bacterial events, however, are typically represented by the emergence of drug-resistant bacterial strains, and the apparent dominance of bacterial EID events is attributed by the authors to their classification of each individual drug-resistant strain as a separate pathogen (Jones et al., 2008)

In humans, fungi account for only 7-9% of emergent diseases (Cleaveland et al., 2001; Woolhouse and Gowtage-Sequeria, 2005), for no emerging diseases in domestic livestock or carnivores, and only 1% of OIE listed animal pathogens.

However, currently *Cryptococcus gattii* is emerging as a novel human fungal pathogen in the North Western United States and Canada (Byrnes, III et al., 2010). In wildlife, Dobson and Foufopoulos found that fungi accounted for 2 /31 (6%) of emerging diseases in their 2001 survey, but this includes the fungal disease chytridiomycosis (*Batrachochytridium dendrobatis*) which is of great significance globally in the serious decline of amphibian populations. Since then white nose syndrome in bats (caused by the fungus *Geomyces destructans)* has emerged and is of great concern (Blehert et al., 2009; Puechmaille et al., 2010) as a new and emerging diseases that has caused more 1 million bat deaths in the USA since 2006, indicating that fungal diseases may be of great importance in terms of wildlife conservation and biodiversity.

Host range

By definition a zoonotic pathogen can infect more than one species or host (one or more animal species and man), and this ability is widely believed to be a significant factor in disease emergence, with the majority of human pathogens (76%) and human EID events (60.3%) being zoonotic, and 90% of emerging diseases in livestock and 100% in domestic carnivores being caused by multi-host pathogens (Cleaveland et al., 2001; Jones et al., 2008; Taylor et al., 2001). However this multi-host phenomenon provides opportunities for surveillance, as non-target species can be utilised or act as potential sentinels (Halliday et al., 2007). Pathogens that infect more than one taxonomic order and that infect wildlife hosts have higher relative risks of emergence than pathogens with a restricted or single host range or that do not have wildlife hosts (Cleaveland et al., 2001). Multihost or generalist pathogens are also commonly associated with disease outbreaks in wildlife (e.g. canine distemper in wild canids, felids and phocids, phocine distemper in northern sea otters (Goldstein et al., 2009), sarcoptic mange in multiple taxa globally (Pence and Ueckermann, 2002), rabies in wild carnivores, including endangered Ethiopian wolf and African wild dog (Cleaveland et al., 2002) but this is not always the case as, for example, white nose syndrome has only been reported in bats (Blehert et al., 2009).

However, the factors that enable a pathogen to have a broad host range are not

clearly understood. High levels of genetic diversity, resulting in many genetic variants that can become associated with different host species, e.g. rabies virus, are likely to be important (Morimoto et al., 1998). A pathogen that infects its host via host cell receptors that are highly conserved across many species may also be a determinant (Woolhouse, 2002). Examples of this are the highly conserved mammalian nicotinic acetylcholine receptor via which the rabies virus gains entry to peripheral nerves in any domestic mammal, and the vitronectin receptor via which the foot and mouth disease virus can infect multiple host species (Baranowski et al., 2001; Holmes and Drummond, 2007). Similarly, the haemagglutinin of influenza A viruses binds to sialic acid (SA) receptors which are found in the tracheal epithelium of many species including pigs and humans and in the enteric epithelium of birds (Nicholls et al., 2008). Even if able to infect multiple species, many pathogens are less infectious to a host that is different to its usual host, a phenomenon referred to as the species barrier (Sansonetti, 2006) – for example the dose of wild-type fox rabies virus necessary to infect cats and dogs has been shown experimentally to be up to a million times greater than that required to infect foxes (Blancou and Aubert, 1997). In contrast, other pathogens may be more infectious or pathogenic in novel hosts, and this can frequently be attributed to the fact that a new, frequently anthropogenic, opportunity has arisen for the host to be exposed to the pathogen. For example, *Toxoplasma gondii* is a widespread pathogen that has recently been recognised as a major cause of mortality in threatened sea otters (*Enhydra lutris nereis*), due to run off from terrestrial freshwater contaminated with domestic felid faeces (Miller et al., 2008). Captive neonatal Pallas cats (*Octocolobus manul*) are also exquisitely sensitive to toxoplasmosis and frequently die in zoo collections; this high pathogenicity is believed to be associated with a lack of any innate immunity in the host species as they have evolved without contact with this pathogen (Brown et al., 2005). Similarly the global amphibian declines driven by chytridiomycosis are believed to be due to the introduction of this pathogen into naïve populations (Walker et al., 2010).

An indirect or vector-borne route of transmission is also associated with a broad host range (Woolhouse et al., 2001). Indirect transmission can involve widespread

contamination of the environment to which multiple hosts are then exposed (e.g. anthrax) and vectors, such as biting arthropods, provide many opportunities for the pathogen to infect multiple hosts e.g. WNV (see also transmission routes below).

In order to make successful species jumps and have the potential to cause epidemics or epizootics, pathogens need to be able to both infect the new host and be transmitted sufficiently within the new host population to persist (Parrish et al., 2008); this is akin to successful colonisation of a new habitat and Dobson and Foufopoulos (2001) liken emerging pathogens to weeds. If $R_0 < 1$ in the new host population and infection relies on continued jumps from the host species, then the pathogen is unlikely to constitute the greatest epidemic threat (e.g. Ebola, vCJD). The features that enable a pathogen to switch or jump hosts are thus of great interest and current focus, especially in relation to the appearance of new human pathogens such as HIV, SARS coronavirus and avian and swine influenza, but also as a feature of important wildlife diseases such as canine distemper (Benmayor et al., 2009; Garamszegi, 2009; Parrish et al., 2008; Woolhouse et al., 2005). In addition, an important animal pathogen, *Staphylococcus aureus* in broiler chickens, has recently been demonstrated to have become an animal pandemic after a human to animal jump only in the last 30 to 63 years, and thus host switching may play a critical role in the development of new anthropozoonoses as well as zoonoses (Lowder et al., 2009).

Anthropogenic factors

The role of agriculture and domestic animals in the origin of human pathogens and evolution of human diseases has been explored recently (Pearce-Duvet, 2006) by close examination of phylogenetic data for several human pathogens that have been linked to domestic animals (measles, pertussis, smallpox, tuberculosis, taenid worms and falciparal malaria). This study challenges the simple hypothesis that the advent of agriculture increased human population sizes to allow maintenance of virulent pathogens and domestic animals provided the source of diseases to humans. It suggests an alternative more complex hypothesis that, rather than domestication *per se*, the ecological changes caused by anthropogenic modification of the environment

associated with agriculture and domestication provide the broader driving force for human pathogen evolution. Although there is strong evidence for a domestic animal origin for measles and pertussis, phylogenetic data do not exclude a non-domestic origin, and the evidence for the other pathogens neither fully supports or refutes a domestic origins hypothesis, with data for tuberculosis and taenid worms suggesting that transmission may occur as easily from humans to animals (anthropozoonotic). In addition, some human pathogens such as tuberculosis, falciparal malaria and dysentery may pre-date the appearance of agriculture, and Pearce-Duvet (2006) suggests that agriculture may have acted by changing the transmission ecology of pre-existing human pathogens, increasing the success of pre-existing vectors leading to novel interactions between humans and wildlife, and, via domestic animals, providing a stable conduit for human infection by wildlife (Pearce-Duvet, 2006).

Whatever the exact mechanism, the socioecological, technological and political factors involved in the dynamics of human diseases are of undoubted importance, and many reviews emphasise the role of anthropogenic social and environmental factors (e.g. (Kuiken et al., 2003). In 1992 the Institute of Medicine identified 6 principal factors as contributing to the emergence of infectious diseases, all of which are fundamentally or potentially anthropogenic:

- Ecological changes
- Human demographics and behaviour
- International travel and commerce
- Technology and industry
- Microbial adaptation and change
- Breakdown in public health measures

These 6 principal factors or causal themes are closely paralleled by factors driving wild and domestic animal disease emergence (Daszak et al., 2000). Analysis of past EID events has confirmed that areas of the world where all these factors are most pronounced can be identified as “hotspots” for future zoonotic disease emergence (Jones et al., 2008); for example Mexico, where the latest H1N1 outbreak originated,

was identified as a significant hotspot, being a country with recent and rapid demographic change, combined with recently intensified livestock production and having a diverse wildlife population which provides a potential pool of new zoonoses (Daszak, 2009). Developing predictive models by identification of such hotspots and focusing on activities within them most likely to pick up EIDs, such as livestock production facilities, or wildlife populations that harbour other zoonoses, has been termed "Smart Surveillance" (Daszak, 2009), and is an obvious method of targeting limited surveillance resources to attempt to identify pathogens before they begin a transitionary journey beyond Stage 1 as described by Wolfe et al (2007), or to attempt to stop them going through further transitionary stages.

It is now widely accepted that climate change is anthropogenic, and this is also implicated in the emergence of many human and animal infectious diseases, especially vector-borne diseases such as WNV (Gale et al., 2010; Ruiz et al., 2010) , bluetongue (MacLachlan and Guthrie, 2010; Purse et al., 2005; Purse et al., 2008) and other arboviruses (Gale et al., 2010; Soverow et al., 2009; Zell et al., 2008) although clear evidence is still lacking (Jones et al., 2008). For example, the recent UK Foresight program (Foresight: Infectious Diseases: Preparing for the Future; Office of Science and Innovation, London) identified the most important factors expected to influence future changes in infectious disease risks as travel, migration and trade, which promote the spread of infections into new populations, but modelling studies on climate change suggested that its effect on infectious diseases would be relatively minor over the next 10 to 25 years (King et al., 2006).

Transmission routes and dynamics

The causal factors associated with the risk of disease emergence (discussed above) are very broad and within each there will be many potential complex mechanisms which could affect pathogen dynamics. The transmission route of pathogens and the dynamics of transmission play a key role in the process of disease emergence, particularly that of zoonotic disease emergence (Cleaveland et al., 2007). Pathogens are transmitted within and between populations directly by close contact (e.g. via inhalation), indirectly (via the environment, food or an intermediate host) or by

vectors. When examining ecological risk factors for zoonotic disease emergence, a framework has been proposed using the concept of samplers (individuals with a high risk of acquiring novel infections) and spreaders (individuals with a high potential for transmitting novel infection onwards within the new host population), and three key steps described (Cleaveland et al., 2007):

- Transmission from animal host to human samplers
- Transmission from samplers to spreaders
- Transmission from spreaders to the general population

An obligate zoonosis, such as rabies or WNV, will not be transmitted from samplers to spreaders, whereas zoonotic pathogens with the potential for onward human to human transmission can affect the general population via spreaders. The risk of transmission at each step will depend on the number of infections in the animal or human source population source (which will depend on the size of the population and the incidence and prevalence of infection), the rate of contact between individuals in each population and the susceptibility of the host population. Transmission risk can therefore be affected dramatically by factors that alter these parameters. For example, the number of infections can be influenced by close proximity of individuals in cities or intensive farming regimes, and poor hygiene and public health. The contact rate between an animal source population and human samplers can be increased by domestication, changing agricultural practice such as encroachment on wildlife habitat, long-distance movement of humans or animals, or ingestion of bushmeat. The susceptibility of the host can be increased by immunosuppression, for example by co-infection with HIV.

As one specific example, for *Borrelia burgdorferi* (Lyme disease), forest fragmentation, reforestation and increased leisure use of the countryside, are all believed to have increased the number of infected animal reservoir hosts and samplers and the opportunities for contact between them, and climatic changes can influence the number of tick vectors (Cleaveland et al., 2007; Gilbert, 2010; Joss et al., 2007; Mavin et al., 2009). Similarly for other examples of zoonoses Cleaveland

et al (2007) identified that emergence is associated with multiple risk factors which operate simultaneously or sequentially in order for a disease to emerge or re-emerge. Although this framework was developed for zoonotic disease emergence in humans, the concept is equally applicable to animal diseases. Appreciation of the complex factors affecting transmission and emergence should help in the targeting of control measures and resources for surveillance, such as the identification of high-risk environments and identification of suitable sentinel animal or human populations.

1.1.3 Surveillance of emerging infectious diseases

The ultimate aim of disease surveillance in both human and animal populations is to identify changes in the infection and/or health status of those populations and to provide rigorous evidence of the absence of disease or determine the prevalence of a pathogen (Salman, 2003). A critical element of surveillance is that an identified response is made on the basis of the data generated, to allow an appropriate action to be taken.

Disease surveillance can be carried out by voluntary or mandatory notification of disease, outbreak investigation, censuses and surveys, or by the use of sentinels, and may be active (targeted) or passive (scanning) in nature. Most human disease surveillance systems are disease-specific and passive and the degree and quality of surveillance varies between countries, leading to a fragmented system with many gaps (Morse, 2007). The development of electronic and internet-based communication systems, such as the Program for monitoring emerging diseases (ProMED) and the HealthMap project (Brownstein et al., 2008), in recent years has led to greater global co-operation.

The impact of EIDs can be minimised through well-prepared and robust public health systems and similar systems developed by the livestock, wildlife and food safety sectors (Merianos, 2007), but effective response depends on preparedness planning, early warning systems and appropriate response capacity. In addition to individual country programmes, international and global programmes do exist in

both the human and animal sector. The International Health Regulations (IHR) provide a legal framework for the international public health response to control human cross- boundary infectious diseases, the Global Outbreak Alert and Response Network of the WHO provides the technical and operational response for the control of global outbreaks, and the Terrestrial Animal Health Code of the OIE (OIE, 2009) aims to assure biosecurity for international trade in terrestrial animals and their products internationally via health measures to be used by national veterinary authorities. However, the quality of pathogen surveillance in animals varies between countries and frequently does not include wildlife (Kuiken et al., 2005). Furthermore, it is recognised that countries that carry out disease surveillance of their wildlife populations are more likely to understand the epizootiology of infectious diseases and zoonoses and are thus better prepared to protect wildlife, domestic animal and public health (Artois et al., 2009; Morner et al., 2002). Greater communication between the fields of veterinary and human medicine, (the so-called "One Health" concept (www.onehealthinitaitive.com), to link pathogen surveillance of wild and domestic animals to public health surveillance both nationally and internationally, will therefore make an important and essential contribution to detection and control of EIDs (Chomel and Marano, 2009; Kuiken et al., 2005; Rabinowitz et al., 2009; Scotch et al., 2009). However, there are limitations to the effectiveness of current global surveillance systems for emerging zoonoses; many capacity gaps and reporting barriers have been identified, and addressing and overcoming these represents a major challenge for zoonosis surveillance worldwide (Halliday et al, 2011).

Use of sentinels for surveillance

The use of sentinels is one form of surveillance that focuses on specific subpopulations to enhance the detection of disease and/or improve the cost-effectiveness of surveillance (McCluskey, 2003). The function of sentinels is generally perceived as being to provide an early warning of pathogen presence for a host species of importance (e.g. man). The aim is to obtain timely information in a cost-effective manner rather than to derive precise estimates of prevalence or incidence of disease (Centers for Disease Control and Prevention, 2008). Sentinels

can be used to address a range of surveillance questions including:

(i) detection of a pathogen in a new area,
(ii) detection of changes in the prevalence or incidence of a pathogen or disease over time,
(iii) determination of rates and direction of pathogen spread,
(iv) testing specific hypotheses about the ecology of a pathogen and
(v) evaluation of the efficacy of potential disease control interventions (McCluskey, 2003).

While at present surveillance of many pathogens involves the target population alone, the broad host range of many important human and animal diseases provides opportunities to exploit a wide range of species for surveillance purposes, including humans themselves. Cleaveland *et al* (2007) suggest that for surveillance of emerging zoonoses specific human sentinel or sampler populations in high risk environments could be used, such as airline crews, groundstaff and frequent flyers (travel hubs), medical staff, immunosuppressed or elderly patients (hospitals), vets, farmers and abattoir workers(farms and animal markets), and bushmeat hunters and consumers (interface habitats). A recent example of high risk human sentinels is the detection of simian foamy viruses in villagers in Cameroon that have direct contact with body fluids from wild primates through hunting and butchering (Wolfe et al., 2004), demonstrating that primate retroviruses are actively being transmitted into human populations. The reduction of bushmeat hunting would therefore have the potential to reduce the risk of emergence of human retroviruses.

Since the use of the coal-miner's canary to detect carbon monoxide (Burrell and Seibert, 1916), it has been recognised that animals have the potential to act as sentinels for human environmental health hazards (Committee on Animals as Monitors of Environmental Hazards, 1991; van der Schalie et al., 1999); for example domestic dogs and the tumours they develop may facilitate identification of environmental carcinogens (Backer et al., 2001). However, animal sentinels appear to have been underused for infectious disease surveillance (Rabinowitz et al., 2005).

Where they are used, animal sentinels can vary from individual animals to herds or larger populations, from animals of the same species to different, more susceptible, expendable or accessible species, and from animals deliberately placed or introduced by man to those already existing in a particular location (Table 1.1).

Table 1.1: Summary of applications of animal sentinels for environmental and infectious hazards. (From Halliday *et al*, 2007)

Type of sentinel	Example	Reference
Individual animal	Coal miner's canary, used to detect the presence of carbon monoxide	(Burrell and Seibert, 1916) (Schwabe, 1984)
Herd/population	Sentinel cattle herds and chicken flocks used to monitor the distribution of arboviruses and their vectors in Australia and the USA	(National Arbovirus Monitoring Program); (Loftin et al., 2006)
Same species	Unvaccinated chickens placed within vaccinated flock to detect HPAI	(Suarez, 2005)
Different, more susceptible species	Feral pigs released into New Zealand to detect the presence of bovine TB – more susceptible than possums; Coal miner's canary (as above)	(Nugent et al., 2002)

Sentinel Application	Example	Reference
Deliberately placed (experimental)	Standard laboratory mice sentinel programmes using outbred mice, sacrificed and tested to detect presence of a panel of rodent pathogens in the core experimental or breeding colony;	(Institute of Laboratory Animal Resources (U.S.). Committee on Infectious Diseases of Mice and Rats, 1991)
	Use of sentinel chickens to evaluate the effectiveness of cleaning an disinfection procedures for eradication of Newcastle Disease	(McCluskey et al., 2006)
In natural habitat (observational)	Wildlife as detectors of DDT and PCB toxicity;	(Hazards et al., 1991)
	Evaluation of white-tailed deer as natural sentinels for *Anaplasma phagocytophilum*, the cause of human granulocytic anaplasmosis;	(Dugan et al., 2006)
	Mesothelioma in pet dogs associated with exposure of their owners to asbestos	(Glickman et al., 1983)
Sentinel unit	Equine premises used to investigate presence of vesicular stomatitis in Colorado.	(McCluskey et al., 2002)

Recognition of the potential use of animals as sentinels is increasing however, and in recent years there are more examples of animal sentinel use for emerging or re-emerging diseases, including the use of badgers to monitor plague in Idaho, USA (Messick et al., 1983), released pigs as sentinels for *Mycobacterium bovis* in New Zealand (Nugent et al., 2002), white-tailed deer as sentinels for *Anaplasma phagocytophilum* (Dugan et al., 2006), coyotes as sentinels for *M.bovis* in Michigan, USA (VerCauteren et al., 2008), sea otters as sentinels for flow of protozoal pathogens such as toxoplasma from the terrestrial to the marine environment (Conrad et al., 2005), and domestic dogs as sentinels for *Borrelia burgdorferi* and West Nile Virus in the USA (Duncan et al., 2005; Resnick et al., 2008). Indeed, the West Nile virus (WNV) outbreak in the USA that started in 1999 provides the most extensive research into the use of animal sentinels in current literature, with the use of the American crow (*Corvus brachyrhycnchos*) in particular, which is especially susceptible to WNV. Mortality in this species preceded both the confirmation of viral activity and the onset of human cases by several months and observation of high crow mortality was used to predict human risk early enough to implement targeted mosquito control and personal protection warnings (Eidson et al., 2001a; Eidson et al., 2001b; Eidson et al., 2001c; Hayes and Gubler, 2006; Johnson et al., 2006; Julian et al., 2002; Komar, 2001; Mostashari et al., 2003; Yaremych et al., 2004), although subsequent analysis has indicated that the American crow may not in fact be the ideal sentinel for WNV as high mortality can be due to other factors not associated with WNV (Ludwig et al., 2010).

Animals have also been identified as potential sentinels for bioterrorism chemical agents (Rabinowitz et al., 2006; Rabinowitz et al., 2008) by providing an early warning system, detecting ongoing exposure risks or by playing a role in maintenance and spread of an epidemic due to intentional release of an agent. In the field of conservation medicine, which focuses on the effects of disease on rare and endangered species and on the functioning of ecosystems, animal sentinels are now also recognised as having the potential to provide insight into the health of an ecosystem (Aguirre, 2009), by using a variety of taxa at different trophic levels and

with different ecological roles, and different spatial and temporal scales. For example, common wild canids such as jackals, coyotes and foxes have been identified having the potential to serve as sentinels for emerging canine vector-borne diseases including leishmaniosis, Lyme disease, heartworm, hepatozoonosis and anaplasmosis that may have a devastating effect on the conservation of other endangered canid species as well as to domestic dog populations (Aguirre, 2009). Marine mammals have also been identified as key sentinels for marine ecosystem health because of their longevity, high trophic level, fat stores that act as depots for anthropogenic toxins and susceptibility to a variety of EIDs such as novel herpesvirus infections and toxoplasmosis (Bossart, 2006).

Therefore sentinels have enormous potential to assist in the continuing battle against EIDs at many levels, and the correct choice of sentinel for a particular application is vital for the success of this type of surveillance.

The sentinel framework

Halliday *et al* (2007) (see Appendix 1) describe a conceptual framework within which the characteristics of different host populations and their potential value as sentinels can be evaluated (Halliday et al., 2007). For a sentinel species or population to be useful for pathogen surveillance it must be under observation and capable of developing a detectable response to a particular pathogen. Sentinels are selected or present themselves because they have attributes that enhance detection of the disease or pathogen and/or improve the cost-effectiveness of surveillance (McCluskey, 2003). In most cases this means that the sentinel (be it individual, population or even species) is more likely to be exposed to, or respond to the pathogen than other individuals, populations or species.

The attributes of an "ideal" sentinel have been listed (Committee on Animals as Monitors of Environmental Hazards, 1991; Komar, 2001), but these have invariably been created with a particular application in mind. For example, for environmental health hazards, CAMEH (1991) gives the attributes as having a measurable response to the agent in question, including accumulation of tissue residues, a territory or home range that overlaps the area monitored, being of sufficient population density

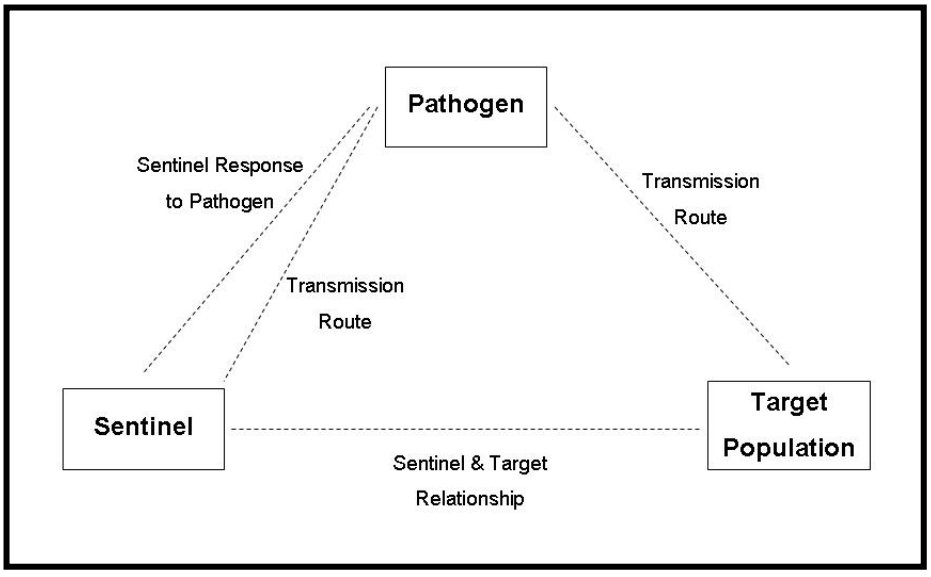
or size to be easily counted and captured, and having a close association with the source of interest.

Another specific example is the assessment of chickens as candidate sentinels for WNV; ideally these would have the attributes of being highly susceptible to mosquito-borne infection, resisting disease and surviving infection in order to produce detectable antibodies, not developing sufficient viraemia once infected to infect biting mosquitoes, and not being able to infect flock mates or human handlers (Langevin et al., 2001). Therefore, an overall framework that incorporates the necessary characteristics of sentinels and their relationship to the pathogen and target population of interest provides a useful tool for evaluating potential sentinel use.

For infectious disease surveillance, a sentinel population must always interact with the pathogen and the target population of concern to which information gathered from the sentinel is applied (Figure 1.1). The key components of the sentinel framework are:

- The pathogen that is under surveillance
- The target population, i.e. the population of concern to which information gathered from the sentinel is applied
- The sentinel population

Figure 1.1: Key components and attributes of the sentinel framework (from Halliday et al 2007)



This framework does not represent the transmission dynamics of the pathogens, but the ways in which the components are associated. The utility of a sentinel for a particular surveillance aim in a particular ecological context thus depends upon (i) the sentinel response to the pathogen, (ii) the relationship between the sentinel and target populations, and (iii) routes of transmission to both target and sentinel populations.

Sentinel response to pathogen

Sentinel response to a pathogen will vary and includes:

- Current infection/presence of pathogen (subclinical infection)
- Seroconversion
- Morbidity
- Mortality

For the first two categories, the sentinel may remain overtly healthy, and some form of sample will need to be obtained from it in order to detect that it has become exposed or infected, such as a blood sample for antibody detection, faeces for parasite oocysts, or tissue samples for bacterial or fungal culture or PCR testing. Morbidity and mortality can represent readily appreciable signals of the presence of a pathogen within an ecosystem (for example, sick or dying crows exposed to WNV). Healthy sentinels that develop a subclinical response will be more useful for investigating maintenance patterns and transmission dynamics of a pathogen, and temporal information, than those who succumb to a pathogen. For example, foxes in Germany have been shown to seroconvert to rabbit haemorrhagic disease virus (RHDV) by ingesting infected rabbits, without developing any disease, but this antibody response declines dramatically after two weeks. Therefore, serosurveillance of foxes to reveal the proportion of the population exposed to this pathogen over the previous two weeks can give insight into the incidence patterns in the sympatric rabbit population (Frolich et al., 1998).

Halliday *et al.* (2007) liken the response of the sentinel to a diagnostic test for the pathogen within the target population, thus having properties analogous to a test sensitivity and specificity. In this sense, the sensitivity of a sentinel refers to its capacity to respond to the presence of the pathogen in the target population (susceptibility to exposure and infection). The specificity of the sentinel response relates to the ease and reliability with which a sentinel response can be interpreted and attributed to a particular pathogen, and this will be closely related to response type. Morbidity and mortality are less specific indicators of the presence of a particular pathogen than specific molecular responses that are observed using a test or assay unique to the pathogen in question, e.g. seroconversion. For example, for H5N1 surveillance, die-offs of wild birds may be due to pathogens other than H5N1 avian influenza virus, thereby reducing the specificity of avian mortality as an indicator of H5N1 presence. Nevertheless, die-offs could trigger an investigation that could identify H5N1 or rule it out. Alternatively, the use of cats to detect antibodies to H5N1 from consumption of infected bird carcases could increase the specificity of the sentinel response.

Therefore, where a different species is used as a sentinel, it is not necessarily that it is more susceptible to a pathogen (as in Table 1.1) but that the pathogen or the response to it is more easily detectable, or the species itself is more convenient or effective to sample or does not have associated conservation issues; in this context the term proxy species is sometimes used (Halliday, 2010).

Relationship between sentinel and target populations

The relationship between sentinel and target populations can be highly variable and incorporates any form of ecological association, including behavioural, epidemiological and spatial aspects. The minimum association that must exist is a spatial one, but this does not necessarily imply spatial overlap, as the pathogen may be spreading on a wave front or emanating from a focal source, and a sentinel population may be selected on the basis of its closer proximity to the focus than the target population. By contrast there may be a very close relationship between the target and sentinel populations, where the sentinels are in fact a subset of the target population, such as unvaccinated sentinel chickens within a vaccinated flock

(Suarez, 2005), or high risk human individuals that are particularly sensitive to infection, e.g. HIV sufferers, or more likely to be exposed, e.g. veterinarians and farmers to animal pathogens (Cleaveland et al., 2007). This type of sentinel should provide a more accurate risk assessment for the target population than one occupying a different ecological niche and pattern of exposure to the pathogen. Finally, the sentinel population may in some cases also be the source of infection for the target population, such as in the surveillance of arthropod vectors for arboviruses (Bryant et al., 2005) (Crabtree et al., 2009).

Transmission routes

The route or routes of transmission of a pathogen to both the target and sentinel population can be important in selection of an appropriate sentinel. If the route of exposure and transmission is the same for both populations, the relative intensity and patterns of exposure of the two populations are important as it may be desirable to select a sentinel with higher levels of exposure that is therefore more likely to show evidence of the presence of a pathogen. For vector-borne pathogens, the feeding preference of the vector may be useful in sentinel selection. For example, domestic dogs are the preferred source of blood meals for *Triatoma infestans*, one of the main vectors of *Trypanosoma cruzi* in Mexico. A comparative serosurvey revealed overall anti-*T.cruzi* IgG prevalence of 16% in dogs compared to 2% in humans (Estrada-Franco et al., 2006) and a strong positive correlation between human and dog seropositivity within the study site. This indicates that, due to this vector feeding preference, dogs may make good sentinels for identifying areas of human seropositivity and monitoring prevalence of trypanosomiasis.

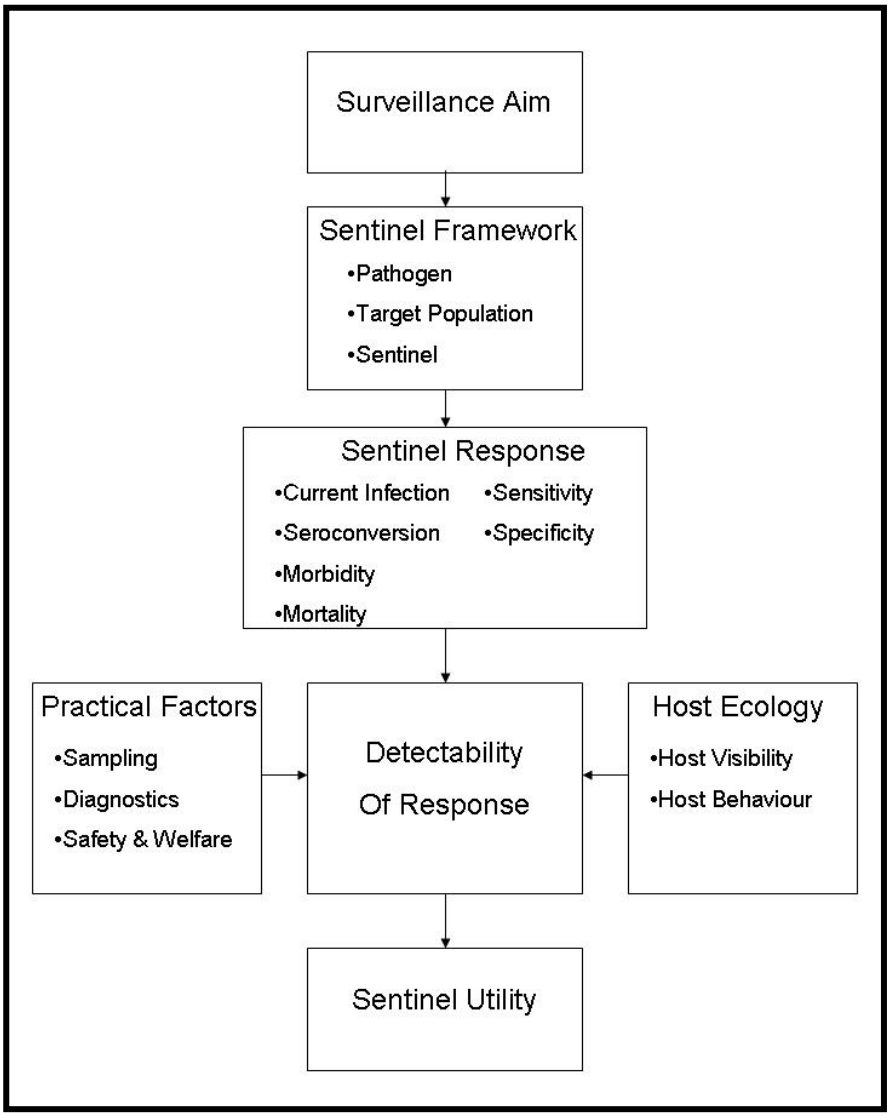
Where the transmission route differs, this may also be utilised in the sentinel context. For example a number of emerging zoonoses, including WNV and HPAI H5N1, can be transmitted via ingestion of infected material (Austgen et al., 2004; Komar et al., 2003; Rimmelzwaan et al., 2006). Therefore carnivore and scavenger species that are exposed through consumption of infected prey may be more sensitive sentinels for a range of pathogens, specifically because this additional route of exposure is not shared with the target population (Cleaveland et al., 2006). In this context, understanding of predator-prey relationships between target and potential sentinel

populations is essential in sentinel selection (see 1.1.4). Humans may also be exposed to pathogens by consuming infected food items, such as bush meat, and therefore those involved could act as human sentinels for the wider population, as exemplified by outbreaks of Ebola virus in western Africa associated with chimpanzee consumption (Georges-Courbot et al., 1997).

Placing the sentinel framework in context

The output of this sentinel framework is the sentinel response. The detectability of this response and overall utility of the sentinel will depend on the nature of the response, other sentinel host factors such as their visibility and behaviour, and practical considerations (Figure 1.2) (Halliday et al., 2007).

Figure 1.2: The sentinel framework in context (from Halliday *et al* 2007)



Sentinel utility can only be assessed by considering both the sentinel framework and the influences of the context in which it would be applied. When considering animals as sentinels, the visibility of a population will be determined by individual's size and morphology, behaviour, distribution and abundance. Detectability of response will depend both on visibility and the nature of the response. For example, lions in the Serengeti National Park are being used as sentinels for canine distemper virus because they are large, active in the day, sought after by tourists to observe, and can exhibit dramatic clinical signs such as grand mal seizures when infected (Roelke-Parker et al., 1996), and thus are highly visible. Information from lion sentinels can then be used to increase detection efforts in other susceptible wild carnivore target populations of concern, including endangered species such as African hunting dogs, to establish the extent and impact of any epidemic and allow possible interventions to be evaluated. As previously mentioned for WNV, widespread morbidity or mortality is more readily detectable than seroconversion or subclinical infection, which require sampling and laboratory analysis. Mortality and morbidity, however, will rely on a reliable network of observers, especially for wild species which may be difficult to detect when dead if small, in remote areas, inaccessible, e.g. inhabiting burrows or rapidly scavenged. Without reliable observation, prevalence data may be highly skewed. For example a study using dead crow decoys to assess possible biases associated with detection and reporting of carcases in surveillance for WNV in the USA, found that the proportion of decoys detected and reported in an urban area was approximately twice and three times that of the rural area respectively, and in a separate study 82% of carcasses had disappeared after 6 days from scavenging (Ward et al., 2006).

Practical difficulties involved in sampling potential sentinels must also be considered, even for a theoretically ideal sentinel in terms of framework and response. It must be logistically feasible in terms of time and cost and safe for personnel to sample sufficient numbers of the sentinel population (Committee on Animals as Monitors of Environmental Hazards, 1991). The effects of sampling on the sentinel population itself must also be considered, in the context of animal welfare and conservation status.

Applications, benefits and limitations of using sentinels

The variability of transmission route and response to a pathogen, heterogeneities in pathogen exposure between different populations and differing relationships between sentinel and target populations all mean that different hosts (animal or human) will vary in their ability to act as effective sentinels in different circumstances (Halliday et al., 2007). By consideration of the sentinel framework, which, although specifically addressing the use of animals as sentinels, is equally applicable to humans, the most appropriate sentinel for a particular context and aim can be determined; i.e. there is no "one size fits all". Sentinels that respond to a pathogen before the target population is exposed or develops disease can be used to provide an early warning of pathogen presence and a predictive signal of risk, thus allowing the opportunity to implement pre-emptive control measures. Other early warning sentinels may be exposed at the same time as the target population, but respond more rapidly (like the coal-miner's canary) – these cannot be used to prevent cases in the target population altogether, but the information they supply can provide advance warning of threat and prioritisation of resources to prevent additional cases. Sentinels that respond in a highly visible way (e.g. morbidity/mortality), are likely to be more useful in an early warning context as data can be more rapidly processed, analysed and acted on than that which requires more lengthy laboratory testing. Early warning sentinels should also ideally respond in a very specific way to minimise the likelihood of false positives and improve confidence in decision making.

Evidence of previous exposure to a pathogen (e.g. antibody presence, or in the case of environmental toxins evidence of tissue accumulation), means that sentinels can also be used retrospectively. This can be used to provide evidence of the timing of introduction of a pathogen and spread through a target population. Where a number of different sentinel populations or locations are sampled, this information can be combined to investigate spatial and temporal patterns of pathogen spread. For example, in the 1993-1997 rinderpest outbreak in Kenya, retrospective serosurveillance of buffalo herds, selected because of their increased susceptibility to the virus, and analysis of age-seroprevalence patterns enabled estimation of the time

of infection of different herds, probable point of entry of rinderpest into the wildlife population and identification of where the pathogen had been, how it had spread, and where it was likely to move to (Kock et al., 1999). The buffalos thus served as sentinels for the larger livestock population, both wild and domestic. For this situation the appropriate sentinel population must develop a response that is persistent over time, have sufficient longevity, and be able to be reliably aged. In some situations the use of sentinels to determine the absence of a pathogen may be key, for example in assessing the success of control measures. Valuable domestic animal populations, such as those used for experimental research, frequently have sentinels applied in such a way (Lipman and Homberger, 2003).

Although sentinels may serve a useful surveillance role in many contexts, there will be situations where their use is not appropriate. The sentinel framework described above, which considers the attributes of a host species needed to identify appropriate sentinel populations, could equally be used to identify characteristics that make potential sentinels unsuitable in a particular circumstance. Although by definition sentinels must be intentionally observed, it is important to distinguish the use of sentinels from scenarios where responses to novel pathogens are noticed. Sentinels cannot be used to provide surveillance for pathogens that are currently unknown. However, it may be that, as a result of greater awareness of the potential of sentinels and improved observation of animal (and human) populations, instances of unusual morbidity and mortality due to the emergence of novel pathogens are more likely to be noticed (Halliday et al., 2007).

1.1.4 Carnivores/predators as sentinels

Carnivores can be subdivided into true predators, such as wild and domestic cats (*Felis* spp), which hunt and consume live vertebrate prey, and scavengers, such as corvids (e.g. crows, magpies, jackdaws, rooks), which opportunistically consume dead prey and predator species. Some species, such as foxes, come into both categories. Depending on the objectives of the surveillance programme, carnivores have the potential to be used as sentinels for other carnivore species by detecting diseases that are shared by both – for example common species of wild canids such

as foxes, coyotes and jackals have been suggested as suitable sentinels for more endangered canid species when assessing disease risks from canine vector-borne diseases (Aguirre, 2009). However, by the nature of their diet, they also have the additional potential of being used as sentinels for diseases present in the animal populations that they consume.

Disease in targeted prey populations, (e.g. rodents, rabbits, ruminants) may be of interest for several reasons. The disease may have primary significance to that target population and carnivore sentinels could provide insight into presence or absence of the disease, and reflect prevalence or indicate incidence patterns, such as the use of foxes to detect patterns of RHDV infection in sympatric rabbit populations (Frolich et al., 1998). Alternatively, interest in the target prey population may be due to their role as a reservoir for disease in other species, including the carnivores themselves and man. Here carnivore sentinels could be useful for providing information on the status or level of a known zoonotic or animal health threat from the target population. For example, coyotes have been used in the USA to as indicators of *M.bovis* presence in sympatric wildlife and domestic livestock (VerCauteren et al., 2008).

Although not yet widely used in veterinary and human disease detection, carnivore and scavenger species possess three main characteristics that suggest they have the potential to act as useful sentinels. First, carnivores are susceptible to a wide range of important human and animal infections. A high proportion (43%) of all zoonotic pathogens infect carnivores (Table 1.2), and thus carnivores represent a suitable taxonomic group for detecting many of the pathogens of concern to human health.

Table 1.2: Important animal host categories for zoonotic diseases (from Cleaveland *et al.*, 2001)

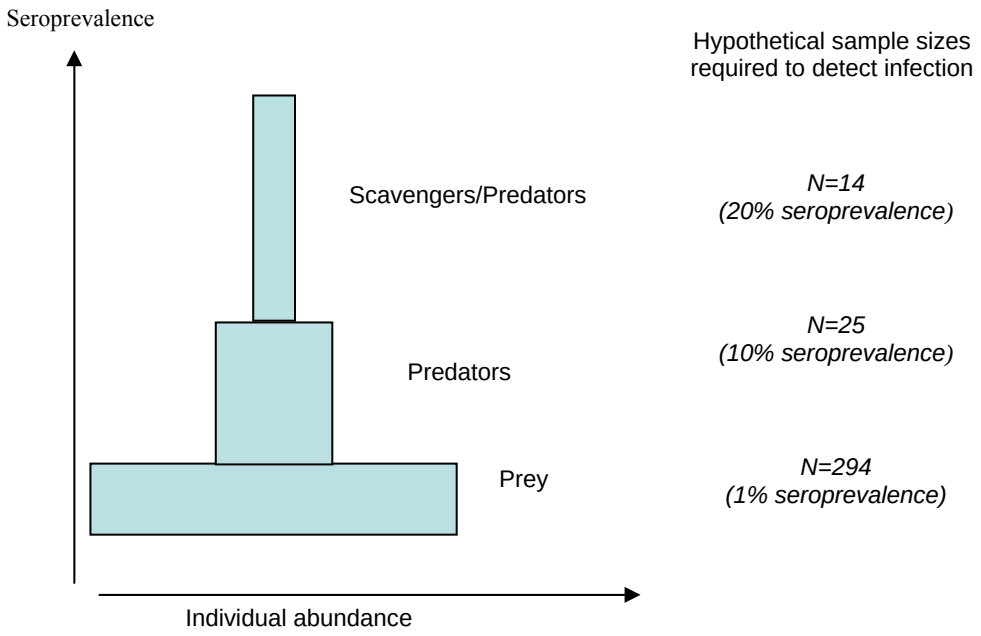
Categories of host infected by pathogen	**Number of zoonotic diseases (n=800)**	**Number of emerging zoonotic diseases (n=125)**
All wildlife species	619 (77.4%)	113 (90.4%)
Birds	82 (10.3%)	23 (18.4%)
Carnivores	344 (43.0%)	64 (51.2%)
Rodents	180 (22.5%)	43 (34.4%)
Marine mammals	41 (5.1%)	6 (4.8%)

Secondly, infection of carnivores through oral ingestion of infected prey may be less pathogenic than infection of other hosts by other routes of transmission, such as inhalation or vector-borne transmission (Austgen et al., 2004; Frolich et al., 1998). High rates of seroconversion (with low morbidity or mortality) in carnivore and scavenger species have been recorded to a wide range of pathogens. Examples include foxes, feral cats, stoats and ferrets, where there is seroconversion to rabbit haemorrhagic disease virus (RHDV) with no evidence of pathogenicity (Henning et al., 1995; Parkes et al., 2004; Philbey et al., 2005), and free-roaming (scavenging) domestic dog populations, in which seroconversion to Ebola virus (Allela et al., 2005), plague (Chomel et al., 1994; Leighton, 2001), Influenza A H5N1 (Butler, 2006) and anthrax (Lembo et al., 2011) have been recorded without evidence for widespread pathogenicity. Experimental infection of cats with WNV has also shown that they develop viraemia but no clinical signs (Austgen et al., 2004). Therefore it may be that carnivores can be used to effectively "sample" the environment for a wide range of pathogens present in different prey species over a long time period, without succumbing to the effects of the pathogen.

Thirdly, a single predator or scavenger will consume material from large numbers of potentially multiple prey species. This is likely to increase the probability of

infection, essentially resulting in a 'bioaccumulation' effect (Cleaveland et al., 2006) whereby a relatively rare infection in a prey species may be detected at a higher prevalence in the carnivore / scavenger species (i.e. a "prevalence pyramid" – Fig. 1.3).

Figure 1.3. Relative seroprevalence of infections that can be transmitted by consumption of infected material



Such bioaccumulation (or bioconcentration) has been suggested as an explanation for the relatively high prevalence of antibodies in domestic dogs to Rift Valley Fever (House et al., 1996), Ebola virus (25%, (Allela et al., 2005), African Horse Sickness (11%, (Alexander et al., 1995)), and anthrax (32%, (Lembo et al., 2011)) in endemic areas of Africa, the high prevalence of antibodies to rabbit haemorrhagic disease (RHDV) in predators and scavengers, particularly foxes, in New Zealand (Parkes et al., 2004), of antibodies to *Coxiella burnetii* in common crows (To et al., 1998), and antibodies to Borna disease in cats (Berg et al., 1998).

A serological survey of domestic carnivores in Canada (Leighton, 2001) demonstrated both the susceptibility to infection and exposure to multiple infections of dogs and cats, detecting exposure to a range of emerging zoonotic pathogens, including *Yersinia pestis (*plague*)*, *Francisella tularensis (*tularaemia*)*, *Rickettsia*

rickettsii (Rocky Mountain spotted fever) and Sin Nombre Hantavirus (Hantavirus pulmonary syndrome). This suggests that both cats and dogs are suitable candidate sentinel species. The Canadian study also demonstrated that sampling domestic carnivores was both more sensitive and more efficient than previous surveys based on collection and culture of rodents and ectoparasites. As an example, previous surveys in the same region for plague collected 20,212 ground squirrels, 62,369 fleas and involved 2,239 inoculations into guinea pigs over 7 years, and had an overall frequency of detection of 1.8%, whereas the serological survey on dogs involved 2.5 months of field work for 2 researchers and detected antibodies to *Yersinia pestis* in 10% of 240 dogs.

Sampling of domestic carnivores has been suggested as a central component of plague (*Yersinia pestis*) surveillance in parts of the USA (Barnes et al., 1988). Similarly, sentinel surveillance using coyotes for bovine tuberculosis in white-tailed deer found that, by focusing on coyotes rather than deer, 97% fewer individuals were sampled and the likelihood of detecting *M. bovis* was increased by 40% (VerCauteren et al., 2008).

However, the use of carnivores as sentinels may not be appropriate in all ecological situations or for all objectives. For example, although carnivores are commonly surveyed as sentinels of local plague activity (Salkeld and Stapp, 2006), one study found exposure to plague in carnivores was so low in situations where epizootics were present in prairie dogs that they gave no useful information regarding habitat-associated foci or temporal changes in plague activity (Brinkerhoff et al., 2009). Further studies are therefore needed to explore in more detail the situations where application of carnivores as sentinels is most appropriate and effective, including analysis of sensitivity, relative prevalence, cost-effectiveness, and how this type of surveillance may be best incorporated into surveillance programmes.

Corvids provide another example of how animals can be used as sentinels for predicting the risk of zoonotic disease. In the last decade they have been the subject of extensive research in North America in relation to their use as sentinels for

predicting human risk from the arthropod vector-borne disease West Nile virus (WNV) (Eidson et al., 2001a; Eidson et al., 2001b; Eidson et al., 2001c; Johnson et al., 2006; Julian et al., 2002). Corvids, particularly American crows (*Corvus brachyrhynchos*) are particularly sensitive to WNV and have a high mortality rate (McLean et al., 2001) (Yaremych et al., 2004). However corvids have also been shown to become seropositive to many pathogens without developing disease, including the zoonotic pathogens *Coxiella burnetii* (To et al., 1998), Borna disease (Berg et al., 2001), *Campylobacter jejuni,* and *E. coli* (Ganapathy et al., 2007) and *Salmonella typhimurium* (Agasi et al., 1967) and can act as natural reservoirs for at least some of these pathogens (Berg et al., 2001; Kapperud and Rosef, 1983; To et al., 1998). The scavenging nature of corvids and the fact that they are frequently found around barns and domestic animal enclosures where they consume animal waste and animal carcases is suggested as a reason why they are more likely to be exposed to certain pathogens such as *C burnetii* (To et al., 1998).

Another benefit of sampling carnivores rather than infected prey is that researchers may be able to be "one step removed" from the potential pathogen. For example, when handling and processing serum or other tissues for the presence of antibody, as it is the response to the pathogen, not the presence of the pathogen itself, that is being detected the risk of exposure to zoonoses is reduced. For example, people conducting surveys of deer mice, the main reservoir host for SNV in the USA, have a significant risk of infection (Mills et al., 1995), but this risk is reduced or eliminated when samples are taken from cats instead (Leighton, 2001) as a serological, rather than microbiological approach can be used.

In summary, carnivores and scavengers are susceptible to a wide range of human and animal pathogens and can act as effective samplers of vertebrate hosts, such as birds, rodents and ungulates, which are important potential reservoirs of many emerging diseases (RIVM (National Institute for Public Health and the Environment), 2004). The likely increased probability of exposure to pathogens through predation and scavenging may also enhance the cost-effectiveness of pathogen detection in carnivore species in comparison with other sentinel hosts, but further work is required to explore the relationships between predators and their prey in terms of

their exposure to common pathogens, how this affects pathogen prevalence in predators and prey and how predators may provide sentinel information.

1.2 Research objectives

The aim of this study is to explore the use of predators as sentinels for selected pathogen-prey-sentinel combinations that have not been previously examined and to evaluate, as a proof of principle, the concept that predators have the potential to act as valuable sentinels for emerging infectious diseases in the United Kingdom. The key questions that the study aims to address are:

- Does sampling predators provide useful information about the presence and prevalence of infection in a given area?
- Does sampling predators provide additional information not available via sampling primary/reservoir hosts alone?
- Is it more cost-effective to sample predators rather than primary/reservoir hosts to detect infection?
- How could predator sampling be incorporated into future surveillance programmes for endemic pathogens or those representing higher level threats?

The study examines particular prey and predator populations in a variety of study sites in Scotland and northern England for serological evidence of exposure to a variety of selected pathogens. Selection of these pathogen-prey-sentinel combinations is discussed in the following chapter. For each pathogen, available serological tests and their applicability to the selected species are explored and novel test methodology developed if necessary (chapters 3, 4, 5 and 6). The relationships between seroprevalence in prey and predator/scavenger species both within and between study sites for the different pathogens and species are explored, and the factors that may affect selection of pathogen, species and site examined (Chapter 7). The practicalities and costs of the study approach area also assessed (Chapter 7), and conclusions drawn as to how effective the study has been in addressing the key questions and objectives and how the information might be applied useful to future

pathogen surveillance (Chapter 8).

Chapter 2: Selection of pathogen-prey-predator combinations

2.1 Introduction

In order to evaluate, as a proof of principle, the concept that predators (carnivores and scavengers) have the potential to act as sentinels for infectious diseases, a number of pathogen-prey-predator combinations that were deemed suitable for investigation needed to be established. These combinations consisted of a selected pathogen, the target prey species population and the candidate predator species population that would act as sentinel. The selection criteria and choices are discussed below.

2.2 Selection of pathogen

There are a large number of pathogens that are of potential interest in terms of causing zoonotic human disease or that affect wild or domestic animals; 177 pathogens have been recognised as causing emerging and re-emerging human diseases, of which 73% are zoonoses (Woolhouse and Gowtage-Sequeria, 2005), 29 pathogens have emerged in domestic livestock (Cleaveland et al., 2001), and 31 pathogens have been associated with new or emerging diseases in wildlife (Dobson and Foufopoulos, 2001). As this study was concerned with the proof of principle that predators can act as sentinels for diseases present in their prey populations, the key focus of the study was the relationship between disease presence and prevalence between predator and prey populations, rather than there being a primary interest in a pathogen or disease itself in either population. Therefore it was important to select pathogens that were believed likely to be found in both predator and prey populations in the selected study sites. The key relationship between the sentinel and the target populations under consideration was that of ingestion of the prey population by the sentinel (see 1.1.4), but by investigating more than one pathogen-prey-sentinel combination, it was hoped that the broader applicability of this approach could also be assessed.

The means by which pathogen exposure or infection would be detected was an important early consideration in the study design. Infection or exposure can be

detected in several ways, broadly divided into:

- Host immune response (cellular or humoral)
- Direct detection of the pathogen in the host
- Indirect detection of the pathogen via the pathological lesions that it produces within the host

Detection of the host's immune response to the pathogen is commonly achieved by measuring antibodies produced by the host and found in serum or other body fluids, and this is known as the humoral response (Washington J.A, 1996). The presence of antibody indicates that the host has been exposed to the pathogen, mounted an immune response, and may or may not be currently infected. For some pathogens, the host cell-mediated immune response can be used to provide a direct visual means of detection in the live animal, for example the intradermal tuberculin skin test for mycobacterial infection commonly used in man and animals (Adams, 2001; Shingadia and Novelli, 2008).

Methods for direct detection of the pathogen range from visual inspection of the external surface of the animal or its internal organs (commonly used for macroparasites, i.e. helminths, tapeworms, arthropods), microscopic examination of tissues or excretory products (e.g. faeces), microbiological culture (e.g. of bacteria and fungi), or molecular techniques, such as polymerase chain reaction (PCR). Indirect detection via pathological lesions can be achieved by gross visual inspection, either of the live animal (e.g. the pathognomonic lesions of Shope papilloma virus in wild cottontail rabbits (Shope and Hurst, 1933), post mortem or at slaughter examination (e.g. the tuberculous granulomas caused by *Mycobacteria* spp. (Kaneene et al., 2006) or microscopically (e.g. vacuolation and presence of fibrils in nervous tissue caused by prion diseases such as bovine spongiform encephalopathy (Scott et al., 1990). However, these methods are invariably followed by direct detection methods to confirm the presence of the suspected pathogen.

However, for this study serological detection of antibodies against a study pathogen

was selected, as this would mean that the same single sample (blood) taken from each animal (prey and predator) could be used for multiple pathogens with a unified test approach, rather than there being a requirement for a variety of tissues depending on the individual pathogen under investigation. Using this approach, it was hoped that seroprevalence data for each predator and prey species could be compared and used to explore the relationships between sentinel and prey populations. The goal of all serological testing is to discriminate between exposed and non-exposed animals, however this is not always possible to achieve with confidence because the serological responses of the two exposure groups usually overlap to some extent (Tyler and Cullor, 1989). Where groups of known exposure status are not available, and/or the test is not validated for use in a particular species, the distribution of results for a continuous variable, (e.g. optical density or antibody titre) can be evaluated to determine if two distinct populations exist. These two populations can then be considered to represent those animals with background reactivity to the test (negative) and those with specific antibodies to the pathogen (positive) (Greiner et al., 1994).

Because serology was the selected as the method of detection, pathogens where direct methods such as microbiological culture (e.g. mycobacteria, fungi) or indirect methods such as the presence of gross lesions are used as the primary means of determining infection were excluded. These methods would have required more time for examination of carcases for specific lesions, collection of particular tissue samples, and/or specific culture techniques. All of these were likely to have differing sensitivities and specificities for each pathogen under study, and may also have required an additional confirmatory step in pathogen detection (indirect methods). In addition, direct contact with lesions or culture of pathogenic organisms would have posed higher zoonotic risks to the researcher than serological examination, and may have required the use of specialised containment facilities (category 2, 3 and 4 pathogens on the Approved List of biological agents, Advisory Committee on Dangerous Pathogens; http://www.hse.gov.uk/pubns/misc208.pdf.) Although all of these issues could be overcome, it was decided that the most efficient and cost-effective use of time and resources for this study was to limit pathogen detection to

serological examination only.

Another group of pathogens excluded from selection were those with intermediate hosts and those with vectors as the main route of transmission, due to the more complex relationships that exist between their different host populations, which would complicate interpretation of data with respect to the evaluation of sentinels. Macroparasites were also excluded as this study was concerned with serological detection of exposure or infection rather than parasitological investigations such as faecal analysis. Serological approaches have been used for some wildlife macroparasites of zoonotic concern, e.g. trichinella in foxes (Davidson et al., 2009), but ingestion of rodents is not believed to be a route of transmission for this parasite.

Vector-borne (e.g. insect or arthropod) or indirectly-transmitted micropathogens have complex infection dynamics that may be non-linear, depending on the biology and ecology of the particular vector, so that transmission potential may change with the prevalence or intensity of infection (Dye and Williams, 1995; Randolph et al., 2002). Such complex and, in many cases, poorly understood relationships might lead to greater difficulty in interpreting infection seroprevalence data and gaining supporting evidence for the bioconcentration concept. Therefore, for the purpose of the proof of principle, it was decided to limit pathogen selection to those micropathogens with direct transmission only.

It was, however, considered important to represent different categories of micropathogen in the pathogens selected (i.e. bacteria, viruses, protozoa), in order to give as broad applicability as possible to the results obtained.

Given the above considerations, the main criteria considered for selection of candidate pathogens were:

- Representation of different categories of micropathogen
- Wide host range and known to infect both prey and predator species
- Known to infect predator species by ingestion, or some evidence that ingestion may be at least one potential route of infection

- Known to be present in the UK
- Serological tests available or described in the scientific literature, at least in some animal species

For the reasons outlined in Chapter 1, selection was also directed at important animal pathogens, either with or without zoonotic potential, that were emerging or re-emerging in animals and/or man, so that any information obtained might be of direct relevance either to the species under investigation, domestic livestock or to human health.

Pathogens were selected on the basis of these criteria after reviewing the relevant literature, and discussions with the funding body and with commercial and government laboratories. There are a large number of potential pathogens that could be investigated, and many pathogens were considered but rejected (Table 2.1).

Table 2.1 Pathogens considered for investigation in this study but rejected

Candidate pathogen	Candidate prey species	Candidate predator species	Main reason for rejection	References
Bacteria				
Salmonella spp	Rodents, birds	Foxes	Detection not reliant on serology	Kapperud, 1998; Kapperud,1983; Refsum, 2002; Handeland,2008; Wales, 2009
Campylobacter spp	Rodents, rabbits, birds	Dogs, cats, badgers	Detection not reliant on serology, no established links between prey and predator species	Meerburg, 2006; {Fernie, 1977; Fernie, 1976; Kwan, 2008; Luechtefeld, 1980; Kapperud, 1983; Palmgren, 1997 Waldenstrom, 2002 ; Colles, 2008; Hughes, 2009
Yersinia spp	Rodents, birds	Foxes	Detection not reliant on serology (*Y.enterocolitica* and *Y.pseudotuberculosis*. *Y. pestis* not present in UK and vector-borne	Kapperud, 1983; Kapperud, 1977; Kapperud, 1975; Kaneko, 1981; Kaneko, 1979; Servan, 1979; Shayegani, 1986; Fukushima, 1991; Nikolova, 2001; Nersesov, 1997;
Pasteurella multocida	Rabbits, birds	Raptors, cats, dogs	Ingestion of infected ducks reported for raptors but no reports in mammalian carnivores. Only reported in UK in wild brown rats, no reports in wild carnivores	Petersen, 2001; Samuel, 2005; Blanchong, 2006; Williams, 1987; Quan, 1986; Curtis, 1980; Webster, 1995
Protozoa and fungi				
Cryptosporidia (C.parvum, C.muris)	Rodents, rabbits,	Foxes, badgers, cats, shrews	Detection not reliant on serology. Wildlife believed to pose low zoonotic risk due to host-adapted genotypes	Fayer, 1986; Chalmers, 1997; Webster, 1995; Sturdee, 1999; Sturdee, 2003; Mtambo, 1991; Feng, 2010
Viruses				
Hantaviruses (e.g.Puumala virus)	Rodents, shrews	Unknown	No definitive evidence of infection in dogs, cats, coyotes. Puumala virus not detected in UK	Jonsson, 2010; Song, 2009; Malecki, 1998; McCaughey, 1996; Bennett, 2010
Cowpox	Rodents	Cats, foxes	Main route of infection is inoculation rather than ingestion	Boulanger, 1995; Boulanger, 1996; Bennett, 1997; Crouch, 1995; Chantrey, 1999 ; Hazel, 2000; Henning, 1995; Muller, 1996; Bennett, 1996
Bornavirus	Rodents, birds	Cats, dogs, foxes, shrews, corvids	No reports in UK in wild prey or predator species	Ludwig, 2000; Berg, 1998; Reeves, 1998; Weissenbock, Okamoto, 2002; Dauphin, 2001; Kinnunen, 2007; Hilbe, 2006;
West Nile Virus	Unknown	Corvids, Dogs	Not reported in UK. Already ongoing surveillance in UK	Komar, 2001; Eidson, 2001; Eidson, 2001; Resnick, 2008

Due to the focus on prey populations, pathogens that have rodents and/or birds as their primary hosts were considered most desirable for selection, and those selected are discussed below.

2.2.1 Bacterial pathogens

Important bacterial pathogens of animals and man that include wild rodents or birds as host species, and can be directly transmitted, include proteobacteria, such as *Salmonella, Campylobacter, Yersinia*, *Pasteurella, and Coxiella,* and spirochaetes such as *Leptospira* species. Although all of these pathogens fulfil many of the criteria for selection (Table 2.1), *Coxiella burnetii* and *Leptospira* spp. were deemed to fit the criteria best and were selected as the most suitable bacterial pathogens for this study. Reasons for rejection of the other pathogens considered are given in Table 2.1.

Coxiella burnetii

Coxiella burnetii is an obligate intracellular proteobacterium belonging to the family *Rickettsiaceae* and is the aetiological agent of Q fever, a worldwide zoonotic pathogen (Woldehiwet, 2004). It has a very wide host range and has been found in most of the animal kingdom (Babudieri, 1959). *C. burnetii* is unique among the family *Rickettsiaceae* in its non-dependence on arthropod transmission and although it can be found in ticks and other arthropods, the main source of infection for domestic animals and humans is exposure to parturient secretions by inhalation of contaminated aerosols (Angelakis and Raoult, 2010; Woldehiwet, 2004). Being an intracellular pathogen, serological testing is mainly relied upon for detection (OIE, 2008b).

Domestic ruminants (cows, sheep, and goats) are the main source of infection for humans, but other reservoirs exist including arthropods, birds and mammals, including small rodents (Burgdorfer et al., 1963; Woldehiwet, 2004). *C burnetii* has been isolated from hares (Marrie et al., 1993), wild mice and rabbits, and Q fever pneumonia in humans has been linked to exposure to wild rabbits (Marrie et al., 1986). In Northern Ireland, a seroprevalence of 9.7% (3/31) was found in rats and 3.2 % (1/31) in mice (McCaughey et al., 2010). Exposure to wildlife has been identified as a risk factor for seropositivity to Q fever in a recent survey of US veterinarians

(Whitney et al., 2009). Cats are now recognised as an important host species and have been implicated in human outbreaks of Q fever (Kosatsky, 1984). Parturient cats are a zoonotic risk (Woldehiwet, 2004) and *C. burnetii* DNA is found in vaginal and uterine samples from healthy cats (Cairns et al., 2007). A Japanese study found a higher seroprevalence to *C. burnetti* in stray cats (41.7%) compared with pet cats (14.2%) (Komiya et al., 2003a), with a greater consumption of wild prey (rodents, birds) suggested as an explanation for the higher seroprevalence in stray animals. Dogs are also a domestic animal reservoir (Woldehiwet, 2004) of *C.burnetii* and human infection has been directly linked to pet dogs (Komiya et al., 2003b).

Thus *C. burnetii* fulfils all the desired criteria listed above and is recognised as an important emerging and re-emerging zoonotic disease, with a recent upsurge of reported cases in humans, domestic ruminants and wildlife in many parts of the world, in particular in the Netherlands, but also in the UK (Amitai et al., 2010; Enserink, 2010; Koch et al., 2010; Lemos et al., 2010; Ruiz-Fons et al., 2008; van der et al., 2010; van et al., 2010; Wallensten et al., 2010). Recent studies in the Netherlands have also indicated that wild brown rats, especially those near farms, may act as true reservoirs for *C.burnetii* (Reusken et al., 2011) and may be implicated in the recent outbreaks in livestock and humans in this country. *C burnetii* is discussed in further detail in Chapter 4.

Leptospira

Leptospira are thin helical bacteria known as spirochaetes. Leptospirosis is a zoonotic disease of worldwide importance, presumed to be the most widespread zoonosis in the world (World Health Organisation, 1999). It is considered to be a re-emerging disease due to the appearance of new serovars and an increasing incidence of human and animal disease in many areas of the world (Higgins, 2004; Levett, 2001) Vijayachari et al., 2008). For example, in Thailand the annual number of reported leptospirosis cases increased from 398 cases in 1996 to 14,285 cases in 2000 (Tangkanakul et al., 2005). *Leptospira* serovars are maintained in nature by numerous subclinically infected wild and domestic animal reservoir hosts that serve as a potential source of infection and illness for humans and other incidental animal hosts (BABUDIERI, 1958; Levett, 2001). Leptospirosis in humans is almost always

acquired via animals (Adler and de la Pena, 2009), although human to human transmission has been recorded through breast-feeding (Vijayachari et al., 2008). Infected animal urine contaminates soil, surface water and water courses and leptospires can then enter the body by direct contact with animal urine or indirectly via these sources, through small cuts or abrasions, via mucous membranes or through wet skin. In humans, a chronic carrier state is very rare, but acute infections can lead to long term sequelae (Adler and de la Pena, 2009). Although long considered an occupational disease, associated with rice farming, mining, sewer maintenance, livestock farming and veterinary medicine, in developed countries leptospirosis is also seen in association with recreational activities such as water sports (Bharti et al., 2003; Vijayachari et al., 2008).

In non-human animals, infection is acquired either directly via the transplacental, haematogenous, or venereal routes or by suckling milk from an infected mother, or indirectly by exposure to urine-contaminated environmental sources (Vijayachari et al., 2008). Therefore, as a host group, carnivores could potentially become infected from their prey either by environmental exposure or directly by ingestion. Ingestion of infected mice has been shown experimentally to induce infection with *Leptospira ballum* in cats (Shophet and Marshall, 1980), however, no other examples of ingestion as a route of infection in animals can be found in the literature.

The most important maintenance hosts of *Leptospira* species are small wild mammals such as rats and mice, which can transfer infection to domestic farm animals, dogs and man (Levett, 2001).These reservoir species have chronic infection of the renal tubules (Twigg and Cox, 1976). Cats have been reported as being rarely affected clinically with leptospirosis, but one survey in Scotland showed that 9.2% of cats were seropositive to *Leptospira* spp. (Agunloye and Nash, 1996). An experimentally induced prey to predator chain of infection with *Leptospira ballum* has been demonstrated from mice to cats (Shophet and Marshall, 1980). Although the prevalence of disease caused by *L. canicola* and *L. icterohaemorrhagiae* has decreased as a result of routine vaccination of domestic dogs against these two serovars, there is evidence that the other serovars such as *grippotyphosa*, *pomona*,

and *bratislava* are becoming more widespread. In addition, *L. australis*, which has caused outbreaks of human disease elsewhere (*e.g.* (Lecour et al., 1989), has recently been recorded as a new infection of phocids in the Moray Firth (Zachariah A, 2005).

In the UK, little is known about leptospiral infection in free-living terrestrial vertebrates, however infection of wild carnivores (fox, badger and mink) with leptospires of the a*ustralis* serogroup has been detected (Hathaway et al., 1983a; Hathaway et al., 1983b). In a 1958 Scottish study *L. ballum* was detected in two Long-tailed field mice (*Apodemus sylvaticus*), one short-tailed or field vole (*Microtus agrestis)* and one bank vole (*Clethrionomys gareolus*), and six strains related to *L.sejroe* and *L. saxkoebing* in *Microtus* and *Clethrionomys* (Broom and Coghlan, 1958). A study in 1969 (Twigg et al., 1969) examined 1668 British wild mammals of 25 species, including predators and prey, for presence of leptospires, both directly and by serum agglutination tests, and found infection in 21 species. Although numbers of each species examined are not given in this study, the percentage infected ranged between 3.2% (weasel) and 47.6% (fox). These results do not state whether detection was by direct microscopy or serology in each case. However, since this study over 40 years ago there appear to have been no similar published surveys carried out on British wildlife, although an MSc project has reported *L.australis* as a new infection of phocids in the Moray Firth (Zachariah A, 2005).

Leptospira spp. were thus selected as suitable pathogens for this study as they fit all the desired criteria listed above, have a specific and well-described rodent reservoir, have a reliance on serological testing as a gold standard (OIE, 2008a), and there is a recognition of leptospirosis as a re-emerging disease (Levett, 2001). Further discussion of *Leptospira* spp is in Chapter 5.

2.2.2 Protozoal, microsporidial and fungal pathogens

Protozoal pathogens include the flagellates (e.g. *Giardia, Trichomonas*), amoeboids (e.g. *Entamoeba*), ciliates, (e.g. *Balantidium*) and the large group of Apicomplexa such as *Babesia, Plasmodium* and coccidians, which include *Eimeria,*

Cryptosporidium and *Toxoplasma* species. Many protozoans that cause human or animal disease are arthropod-borne (e.g. *Plasmodium, Babesia, Trypanosoma, Leishmania* spp.) and many have intermediate hosts (e.g. *Toxoplasma*), which ruled them out of this study. Coccidian microparasites such as *Eimeria* and *Isospora* spp. tend to be species specific, and these and the flagellates and amoeboids are generally diagnosed by detection of the parasite itself, rather than by serological testing for antibodies. Therefore these protozoans were also excluded from selection for this study. *Cryptosporidium parvum* and *C. muris* were considered as candidate pathogens but were rejected largely due to reliance on faecal rather than serological test methodology (Table 2.1)

Encephalitozoon cuniculi

Microsporidia used to be classified with the protozoa due to their lack of mitochondria, but are now recognised as being closely related to fungi and include pathogens such as *Encephalitozoon* species (Didier, 2005; Keeling et al., 2000). Three strains of *E.cuniculi* have been established using immunological and molecular methods ; strains I, II, and III, also named "rabbit strain," "mouse strain," and "dog strain", respectively, according to the number of short repeats in the ribosomal internal transcribed spacer region and due to the species from which they were originally isolated (Didier et al., 1995). Rabbits have thus far only been reported to be infected with the rabbit strain under natural conditions, and confirmed human cases have been with "dog" and "rabbit" strains, but a strict host specificity of the strains has not been demonstrated under experimental conditions (Mathis et al., 1997).

Encephalitozoon cuniculi is an obligate intracellular microsporidian protozoan that causes an important emerging disease in both man and animals (Wasson and Peper, 2000) (Halanova et al., 2003). Human infection occurs in immunosuppressed individuals and *E.cuniculi* has emerged as a zoonosis with the advent of HIV/AIDS (Didier et al., 1996; Fournier et al., 2000; Kodjikian et al., 2005; Rossi et al., 1998; Schwartz et al., 1994; Snowden et al., 1999). *E.cuniculi* is the most widely studied mammalian microsporidian and infection in domestic and laboratory rodents and

rabbits is well documented (Wasson and Peper, 2000) (Thomas et al., 1997). It is also known to infect shrews, sheep, goats, pigs, horses, and nonhuman primates (Canning and Lom, 1986). In animals the main target organs are the central nervous system and the kidney, which can result in a granulomatous encephalitis and nephritis. Infection in rabbits and rodents is frequently subclinical, but infected animals can also exhibit neurological signs such as head tilt, paralysis and seizures, and renal failure. In carnivores, encephalitozoonosis is a severe neurological disease of neonatal animals, due to transplacental infection from chronically infected dams (Wasson and Peper, 2000), and is a major cause of economic losses in farmed blue foxes in Scandinavia (Akerstedt, 2002). In the horse, however, the main clinical signs are abortion and stillbirth (Patterson-Kane et al., 2003).

In humans, symptoms in immunosuppressed patients include fever, abdominal pain, nausea, vomiting, renal failure, ocular problems such as keratoconjunctivitis, pneumonitis, and neurological signs, and the organism can be recovered from urine, kidney, CSF and lung (Mathis et al., 2005). Despite the zoonotic potential and opportunistic nature of *E.cuniculi* being well recognised (Mathis et al., 2005) (Deplazes et al., 1996), the exact source of human infections is unclear. Some human patients infected with strain I (rabbit strain) recall exposure to rabbits (Mathis et al., 1997) (Weber et al., 1997), and some of those infected with strain III (dog strain) owned dogs (Didier et al., 1996; Teachey et al., 2004), but infection in the respective animals was not proven. As the spores of *E.cuniculi* are highly resistant in the environment (Li et al., 2003), direct contact with infected animals is not necessary for transmission to occur. Waterborne infection has been proposed but the strains so far known to infect humans (I and III) have not been found in surface water to date, although one isolation of strain II is reported (Mathis et al., 2005).

Although commonly described in laboratory rodents, including in the UK (Gannon, 1980), *E. cuniculi* prevalence in wild rodents is poorly described, although it has been found in a free-ranging rat in Switzerland (Muller-Doblies et al., 2002). *E.cuniculi* occurs widely in domestic rabbits in the UK, with one study finding a

seroprevalence of 52% (Keeble and Shaw, 2006). However, only one study reports the finding of *E.cuniculi* in wild rabbits in the UK, which came from the Pentland hills in Scotland (Wilson, 1979).

Natural infection with *E. cuniculi* has been reported in domestic dogs in many countries including South Africa, Tanzania, and the United States (Mathis et al., 2005) and three cases have been reported in domestic cats (Canning and Lom, 1986). A study in 1989 in the UK in stray dogs recorded a seroprevalence of 13.3% (Hollister et al., 1989). *E. cuniculi* is a major endemic disease problem in farmed blue foxes in Scandinavia, with infection attributed to ingestion of food contaminated with infected rodent urine or faeces (Canning and Lom, 1986). However, there is limited information on the disease in wild carnivores. *E. cuniculi* has been detected in the brain of a wild hand-reared red fox in the UK (Wilson, 1979), and in captive wild dog pups (*Lycaon pictus*) (Van Heerden J. et al., 1989). Seroprevalences of 12% in wild arctic foxes and 8% in feral mink have been reported in Iceland (Hersteinsson et al., 1993). In the Czech Republic, *E.cuniculi* has been detected in the brains of 2/61 (3.28%) martens and in one European otter (Hurkova and Modry, 2006).

Encephalitozoon infection has also been reported in chickens (Reetz, 1993) and psittacine birds (Poonacha et al., 1985), and although later molecular techniques have revealed that some reported psittacine infections are in fact caused by *E. hellem* (Black et al., 1997; Phalen et al., 2006), *E. cuniculi* has recently been confirmed in the cockatiel (Kasickova et al., 2007). The potential for other carnivorous avian species such as raptors, corvids and gulls to become infected and act as zoonotic reservoirs has not yet been investigated.

Although definitive diagnosis of *E.cuniculi* relies on demonstration of the organism by direct or molecular techniques, screening for infection relies mainly on serological testing (Mathis et al., 2005). Therefore *E.cuniculi* also fits all the selection criteria for this study and was chosen as suitable for investigation. Further discussion of *E.cuniculi* is in Chapter 6.

2.2.3 Viral pathogens

Several viral pathogens were considered as candidate pathogens for study, including hantaviruses, cowpox, bornavirus and West Nile Virus (Table 2.1), however these were rejected and rabbit haemorrhagic disease virus (RHDV) was selected as a suitable pathogen for study.

RHDV

RHDV is a calicivirus and an important rabbit-specific pathogen that apparently appeared as a new disease in the UK in 1992 (Fuller et al., 1993). It was first described in China in 1984 where it caused mortality rates of between 80 and 99% in domestic rabbits, and subsequently spread worldwide. Outbreaks in wild rabbits in the UK were first reported in 1994 (Forrester et al., 2009) and epidemics with high mortality rates were observed but were random in distribution and did not appear to have uniform spread. It has been shown that up to 60% of wild UK rabbits have antibodies to RHDV (Trout et al., 1997) Recent serological and molecular epidemiological studies indicate that RHDV circulated as an apparently avirulent virus throughout Britain more than 50 years ago, and, based on molecular phylogenetic analysis of British and European RHDV sequences, it has almost certainly circulated in Britain and Europe for centuries (Moss et al., 2002). It is now postulated that circulating avirulent strains may provide immunity against the introduced epidemic strains that can cause high mortalities in naïve young rabbits (Forrester et al., 2009). A recent Australian study has shown that antibodies against RHDV in fox serum can serve as an index of the occurrence of rabbit haemorrhagic disease in rabbit populations (Philbey et al., 2005). Feral cats, mustelids, gulls and raptors have also been shown to be seropositive for RHDV in New Zealand (Parkes et al., 2004). House mice and brown kiwis have also been shown to seroconvert when experimentally infected with RHDV (Buddle et al., 1997). There has been no evidence for human seroconversion to date. As the disease is rapidly fatal in rabbits, with death occurring in up to 95% of infected animals within 1-3 days (Cancellotti and Renzi, 1991), the serological status of alternative (surviving) hosts, such as foxes and corvids, can provide a particularly useful indicator of where and when outbreaks have occurred. Although currently believed to be a rabbit-specific pathogen and not a zoonosis, RHDV can have a devastating effect on rabbit populations and therefore

it still fits the selection criteria for the study.

2.3 Selection of prey species

The target prey populations in the selected sentinel frameworks under investigation were those species that are hunted or scavenged and ingested by the sentinel carnivores. In the UK the predominant diet of the majority of wild mammalian and avian carnivores such as the red fox, mustelids (e.g. weasel, stoat, mink), and birds of prey are small mammals such as wild rabbits and rodents, and garden and other passerine birds (Mammal Society, 2011; RSPB, 2010) thus these were the initial focus of prey species selection. Domestic cats also target these species in large numbers as prey (Churcher and Lawton, 1987; Woods et al., 2003). However, although garden and other passerine birds were initially considered a suitable prey species, many species are protected under law, and the only access would be via leg ringing exercises, which were found to be unlikely to occur in the selected study sites (see 3.1) (David Noble, British Trust for Ornithology, personal communication). Therefore these bird species were excluded as target prey species.

Fish, amphibians and invertebrates are also consumed by some wild mammalian and avian carnivores. For example foxes will eat amphibians, earthworms and other insects, especially as juveniles (Harris, 1981; Soulsbury et al., 2008), and otters and ospreys consume mainly fish. Although amphibians have been shown to harbour *Leptospira* species (BABUDIERI et al., 1973; Diesch et al., 1966; Gravekamp et al., 1991), that are also found in wild mammals in the same vicinity (Diesch et al., 1970), no reports could be found of infection of fish, amphibians or invertebrates with the other three selected pathogens, or potential transmission to mammals, and they were therefore not considered further as selected prey species.

By focusing on the natural mammalian prey of the majority of UK carnivores, the criteria considered for selection of prey species were:

- Known to be able to be infected with at least one candidate pathogen

- Known prey item for several predator or scavenger species
- Common species with wide geographical distribution across varied habitats, so likely to be found in selected study sites
- Known methodology for capture
- Not of endangered or vulnerable conservation status or protected by UK law (e.g. water vole, dormouse)

Endangered or protected animals were not considered because of the necessity for obtaining appropriate licences and permission, but, mainly for conservation and ethical reasons, it was not considered justifiable to trap or kill any such species for the purposes of establishing the proof of principle for this study.

Using these criteria, the following prey species were selected:

- Field vole (*Microtus agrestris*),
- Bank vole (*Myodes glareolus*),
- Wood mouse or long-tailed field mouse (*Apodemus sylvaticus*),
- Rabbit (*Oryctolagus cuniculus*)

Field voles, bank voles and wood mice are the three commonest wild rodent species in the UK (Mammal Society, 2011) and are not protected by law. These species can be obtained by targeted live trapping (Burthe et al., 2008; Hazel et al., 2000). Rabbits are an abundant prey species with an estimated population in 1995 of 37.5 million (Harris et al., 1995), although current numbers are believed to be above 40 million. They are considered a pest species and are not protected under law (http://www.defra.gov.uk/foodfarm/landmanage/pests.htm), and thus can be obtained via routine pest control practices such as shooting.

It was recognised that shrews (*Sorex* spp.) may be caught occasionally during the rodent trapping process. These species are not endangered in the UK but are

protected in the UK under the Wildlife and Countryside Act 1981 with respect to certain methods of trapping and killing, (http://www.snh.gov.uk/protecting-scotlands-nature/protected-species/which-and-how/mammals/shrew-and-vole-protection/) and a licence is required to trap them for scientific or educational purposes. However, as long as trapping is not targeted or intentional for this species, small numbers accidentally trapped are tolerated without the risk of prosecution, as long as measures are taken wherever possible to prevent any harm, such as checking traps frequently (http://www.snh.org.uk/pdfs/licences/TrapShrews.pdf). Shrews are insectivores and are generally not believed to be predated upon heavily by wild carnivores, as they have a noxious taste (Mammal Society, 2011). There is also evidence that shrews themselves predate on other rodents such as voles (Eadie, 1952). Although domestic cats are known to catch but are not believed to consume shrews, this common belief may be overstated as shrew remains can frequently be found in the stomachs of domestic cats (Nader and Martin, 1962). Shrews are known to be a reservoir of leptospirosis (Kositanont et al., 2003) and thus if found dead in traps would be included in the study, but otherwise released. Occasionally, other less common species of rodent may be encountered in traps targeted for voles and wood mice (e.g. yellow-necked mouse, harvest mouse), and these would be sampled, or released if a protected species (e.g. water vole, dormouse).

2.4 Selection of predator and scavenger species

In order to provide supporting evidence for the bioconcentration concept and utility as potential sentinel species, predators that are towards the top of their particular food chain were selected. The mammalian species belonging to the Order Carnivora in the UK are the red fox (*Vulpes vulpes*), the mustelid species otter (*Lutra lutra*), badger (*Meles meles*), stoat (*Mustela erminea*), weasel (*Mustela nivalis*), polecat (*Mustela putorius*), pine marten (*Martes martes*) and mink (*Neovison vison*) and the Scottish wild cat (*Felis silvestris grampia*) (Harris and Yaldon, 2008). Domestic cats that hunt, and feral cats are also important predators of rodents and small birds in the UK (Woods et al., 2003). All these carnivores are at the top of their food chain, i.e. have no natural predators of their own. Marine species such as seals (Common seal, Grey seal) and cetaceans (e.g. dolphins and porpoises) are also top predators but are protected by law and difficult to access in the marine environment and were not

considered for this study.

Sampling of wild carnivores can be achieved from either live animals (commonly under anaesthesia and followed by release or euthanasia), or after death. Means of gaining access to live wild carnivore species include targeted live trapping or opportunistically through wildlife rescue centres. Live domestic carnivores (e.g. cats, dogs, ferrets) can be more easily accessed directly for sampling via their owners or via veterinary practices, both with informed owner consent. Alternatively, wild animal carcases can be obtained either via lethal pest control programmes (shooting) or opportunistically, for example by collecting road traffic casualties or via wildlife rescue centres. Opportunistic methods are inherently unreliable and might result in insufficient specimens being collected from selected study sites. Targeted live trapping is a well-recognised method of catching carnivores, particularly for pest control, and there are numerous suppliers of traps suitable for all species, including feral cats, (e.g. http://www.sasa.gov.uk/). The necessity to set traps and check them at least every 24 hours means that these methods can be labour intensive when used for research purposes, and subsequently require either anaesthesia or euthanasia of the captured animal in order to obtain a blood sample. For scientific studies, such as various surveys of fox disease in the UK (Delahay et al., 2007; Hamilton et al., 2005; Richards et al., 1995; Smith et al., 2003), sampling of carcases of wild carnivores is more commonly employed, with these obtained via routine pest control methods such as shooting.

Mustelid species are not routinely controlled by man (except mink) and were considered unlikely to be available in any significant number to be useful for the purposes of the study. The Scottish wildcat is the only wild feline present in the UK, but is critically endangered with only an estimated 400 individuals left (Scottish Wildcat Association, 2011) and is protected under the Wildlife and Countryside Act 1981. Domestic dogs were considered unlikely to consume rodent or rabbit prey to any extent, although no published studies on the extent of predation by domestic dogs on rodents, rabbits or birds in the UK could be found. Some working breeds, such as terriers used to control rat populations, and dogs allowed off-lead to hunt

wild rabbits may be an exception to this.

Amongst avian species the raptors, or birds of prey (eagles, hawks, falcons and owls), of which there are 17 species resident in the UK (RSPB), occupy the top predator niche. Some raptors are also scavengers, such as buzzards and eagles. Migratory raptors, such as osprey (*Pandion haliaetus*) and Montagu's harrier (*Circus pygargus*) were not suitable for this study as, due to their migratory habits, their prey would include populations not present in the UK. Most species of raptor are protected by law in the UK under the Wildlife and Countryside Act 1981. Although several species (e.g. buzzards, peregrine falcons, owls, golden eagles) are captured routinely for ringing by government agencies such as the Forestry Commission and charities such as the Royal Society for Protection of Birds (RSPB), which would provide opportunities for blood sampling, this is mostly at the chick stage (David Anderson, Forestry Commission conservation manager, personal communication), where serological response would have been difficult to assess.

The main scavenger species in the UK are the corvids; carrion crow (*Corvus corone corone*), hooded crow (*Corvus cornix*), and magpie (*Pica pica*). These corvids are omnivorous but include dead animals in their diet (RSPB, 2010). Rodents are not thought to make up a significant part of their diet (RSPB, 2010), but larger species, including those that are commonly killed by road traffic such as rabbits, carnivores and game birds may be eaten to a greater or lesser extent depending on location. With the exception of the chough (*Pyrrhocorax pyrrhocorax*), corvids are not protected in the UK and are commonly subject to control by man. Therefore they could be considered a potential sentinel species for this study.

The criteria used for selection of predator and scavenger species were:

- Reported to have shown serological evidence of infection with at least one candidate pathogen
- Known to consume by predation or scavenging at least one selected prey

species

- Common species with wide geographical distribution across varied habitats, so likely to be found in selected study sites
- Ease of capture/accessibility for sampling
- Known methodology for capture
- Availability of carcases from other sources
- Not of endangered or vulnerable conservation status, or protected by UK law

Based on these criteria and after discussions with gamekeepers, pest control operators, conservation personnel, and veterinary surgeons that could participate in the acquisition of samples, the selected predators were:

- Fox (*Vulpes vulpes*)
- Corvids (Crow (*Corvus corone corone*); magpie (Pica pica))
- Domestic cat (*Felis catus*)

Foxes and corvids would be obtained dead via ongoing pest control programmes, and cats would either be sampled by the researcher or samples obtained from other veterinary surgeons with informed owner consent.

2.5 Summary of selected combinations

In summary, the four selected pathogens and their prey and sentinel combinations are given in Table 2.2.

Table 2.2: Summary table of selected pathogen-prey-sentinel frameworks

Pathogen	Prey	Sentinel	Site	References
Coxiella burnetii (Proteobacteria)	Rodent, rabbit	Fox, cat, crow	Rural, semi-urban	McQuiston & Childs (2002); To *et al.,* (1998); Komiya T *et al.,* (2003); Matthewman *et al.,* (1997)
Leptospira species (Spirochaete)	Rodent	Fox, cat	Rural, semi-urban	Hathaway *et al.,* (1983); Agunloye & Nash (1996)
Encephalitozoon cuniculi (Microsporidian)	Rabbit, rodent	Fox, cat, crow	Rural, semi-urban	Halanova *et al.,* (2003); Hollister *et al.,* (1989); Wasson & Peper (2000)
Rabbit Haemorrhagic disease (Calicivirus)	Rabbit	Fox, cat crow	Rural	Parkes *et al.,* (2004); Philbey *et al.,* (2005) ; Henning *et al* (2006)

Selection of the sites used to carry out sampling of the selected prey and predator species, and the results of the animal collection and sampling are described in Chapter 3.

Chapter 3. Study site selection and prey and predator sample collection methods

3.1 Selection of study sites

In order to study the selected pathogen-prey-sentinel combinations, appropriate study sites were identified. Candidate study areas were assessed on the basis of:

- Presence of candidate prey and sentinel species
- Variation in geography and habitat types
- Presence or likely presence of at least one candidate pathogen
- Accessibility for sampling (including access to veterinary practices)
- Presence and cooperation of local landowners and gamekeepers
- Logistic feasibility and accessibility

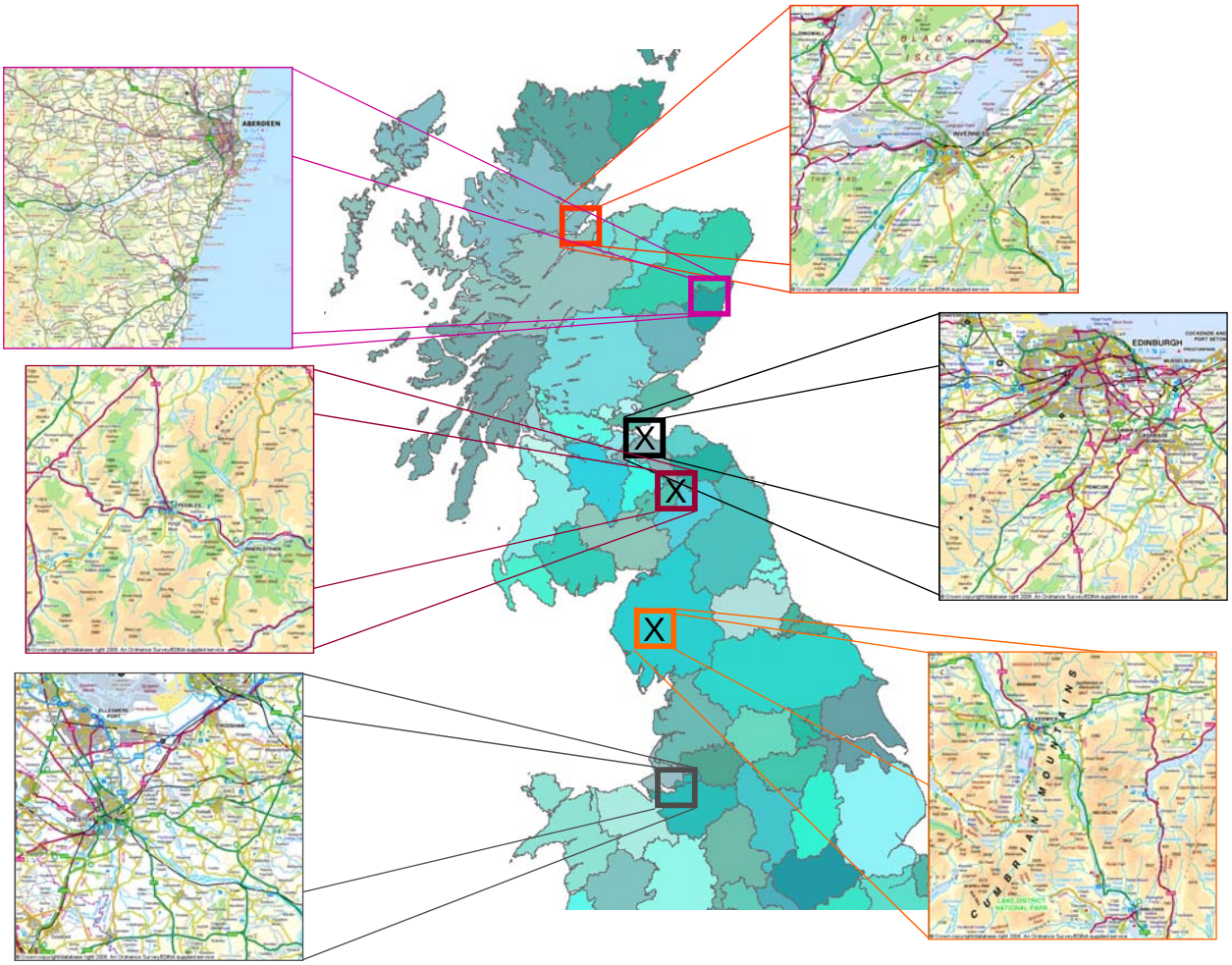
For this particular study the urban environment was not selected because routine lethal control of foxes is not carried out in the cities and towns most convenient for the study (The Scottish Government, 2007), and the selected rodent species are less common than in the rural environment (Harris and Yaldon, 2008), which would limit the prey-carnivore combinations.

In addition to these criteria, practical and cost issues for each site were also considered, such as travel and accommodation costs, travel time, time to return with samples for storage and laboratory processing, and availability of local on-site facilities for storage and processing of samples if the site was remote from the main laboratory and storage facility. These were considered firstly because of the implications for sample quality. For example, the time from taking a blood sample to centrifugation to separate serum will affect the quality of the serum collected due to increasing risks of haemolysis with increased time. Blood needs to be left for at least two hours to clot if serum, rather than plasma, is being used, and once the clot has formed ideally serum should be separated straight away (Kerr, 1989), but this is often not possible in the field. Storage conditions and temperature before separation

will also affect serum quality, and ideally samples need to be kept chilled at 4°C during storage before separation (Kerr, 1989). Secondly, these factors were considered in terms of evaluation of the cost-effectiveness of the study. For this evaluation, factors such as researcher time and effort spent to collect and process samples for each of the selected species were semi-quantitatively estimated (see Chapter 7).

Initially six possible areas in Scotland and northern England were identified using these criteria (Figure 3.1)

Figure 3.1 Map of Northern England and Scotland indicating initial study sites considered for the study (with filled boxes indicating sites chosen (see below)).



These six areas were:

- Scottish Borders – via personal local contacts with the Forestry Commission

allowing access to large areas of forest in the Tweed Valley;

- Cumbria – estates owned by United Utilities Ltd , via Veterinary Laboratory Agency (VLA) Penrith, who receive wildlife samples from these estates via the United Utilities wildlife conservation officer;
- Pentland hills – local to the main laboratory, storage facility and primary workplace and thus quickly and easily accessible;
- Aberdeen area – via contacts with the Macaulay Land Research Unit (James Hutton Institute) who were planning rodent trapping studies to investigate tick-borne disease;
- Inverness/Moray Firth area – via contact with a practising veterinarian with a particular interest in suspected Bornavirus (initially considered as a potential pathogen) in cats in the area, and as a result of a recent study describing *Leptospira australis* serovars in phocids in the Moray Firth (Zachariah A, 2005);
- Cheshire farmland – via colleagues at the National Centre for Zoonosis Research, University of Liverpool, who have ongoing rodent trapping studies in this area.

After assessing these potential sites, three were eliminated for the following reasons:

a) It was apparent that it would not be logistically possible for one person to conduct trapping studies and coordinate and collect carcases from all six sites, plus be able to process and freeze samples effectively in the required time period;

b) Site too geographically distant (Aberdeen, Inverness, Cheshire) from an Edinburgh base to be cost and time effective;

c) For the potential Aberdeen area site, only live trapping with release of rodents was planned, and the Aberdeen collaborators felt there would be insufficient amount of spare serum samples available to donate to this study to conduct serological tests on four pathogens.

Therefore the three final study areas selected were:

1) Borders. Area around Stobo village. Commercial forest with clearcut areas and some farmland. Rural.

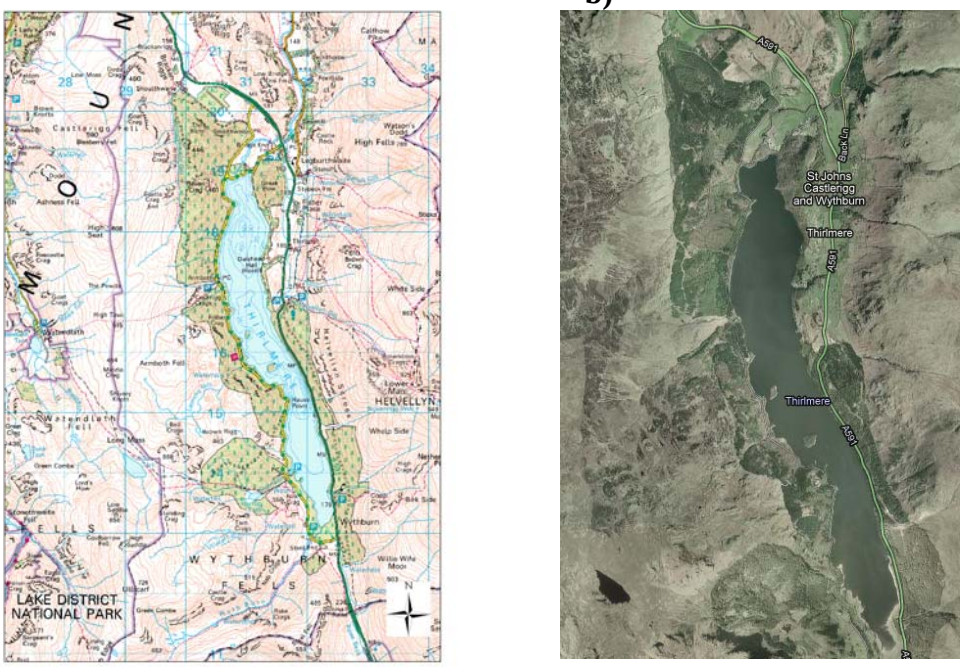
Figure 3.2 General map of borders area a) Ordnance Survey b) Satellite image

a) **b)**

2) Cumbria. Thirlmere. Lakeside and hill farmland plus commercial and native forest. Rural.

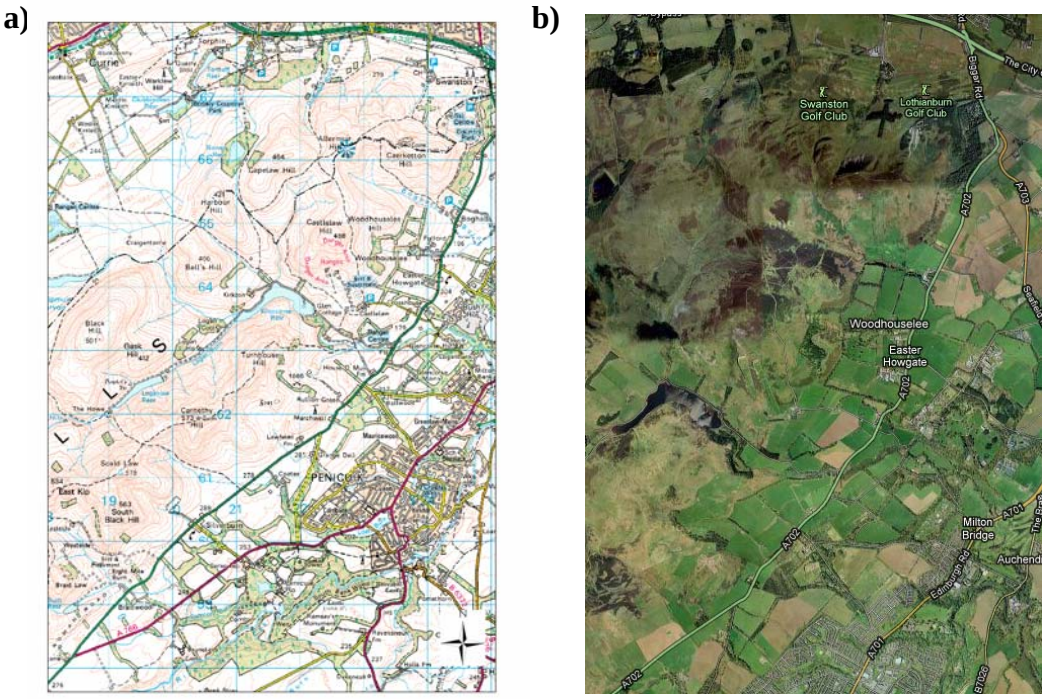
Figure 3.3 General map of Cumbria area a) Ordnance Survey b) Satellite image

a) **b)**



3) Pentlands. Area surrounding the Royal (Dick) School of Veterinary Studies. Mixed farmland and low open hills with close proximity of a town (Penicuik) and Edinburgh city boundary. Rural and semi-urban.

Figure 3.4 General map of Pentlands area a) Ordnance Survey b) Satellite image



Study areas in the three locations (Pentlands, Borders, Cumbria) for prey and predators were initially designated as comprising an area of approximately 78.5 km^2 (a circular area with diameter 10km). However, although prey trapping sites were all accommodated within an area of this size in each study area, logistics of collecting fox and cat samples meant that the final study area for both prey and predators encompassed approximately 176.6 km^2 in the Pentlands, 314 km^2 in the Borders and 380 km^2 in Cumbria. Home ranges of rodents are likely to vary depending on species and population density but examples are 230-1200 m^2 for wood mice, 270-1700m^2 for bank voles and 200-700m^2 for field voles (Mammal Society, 2011). Home ranges of foxes vary widely depending on habitat richness (Goszczynski, 2002; Lucherini and Lovari, 1996) and territory sizes are known to vary at least 200-fold (Macdonald and Bacon, 1982) from as small as 10 hectares (0.1 km^2) in suburban Oxford to 400 - 600 hectares in the Welsh hills and Holland (Dekker et al.,

2001; Lloyd, 1980), more than 1000 hectares in the northern fells of the UK and 2000 hectares (20 km^2) in Canada (Voigt and Macdonald, 1984). Home range for domestic suburban pet cats have been reported as less than 1 hectare (Bradshaw, 1992) or 0.02-27.93 hectares (mean 7.89 ha) (Barratt, 1997). Feral or free-ranging domestic cat (*Felis catus*) home ranges vary about 200-fold depending on habitat, from 0.84 hectares ($0.0084km^2$) to 112 hectares ($1.12km^2$) (Macdonald and Apps, 1978) (Fitzgerald and Karl, 1986; Liberg, 1980; Turner and Mertens, 1986; Warner, 1985). Corvid home ranges are also likely to vary depending on habitat, but have been described in American crows as $6.4km^2 – 9.6km^2$ (Yaremych et al., 2004).

3.2 Licensing

A Home Office Project Licence (PPL) and personal licence (PIL) under the Animals (Scientific Procedures) Act 1986 were obtained, to authorise restraint, anaesthesia and blood sampling of live wild rodents, foxes, corvids and cats. The study was also approved by the local (Royal (Dick) School of Veterinary Studies) and institutional (University of Edinburgh) Ethical Review Process.

3.3 Sample collection

3.3.1 Wild rodents

Trapping sites for wild rodents were selected within each study site using the following criteria:

- Habitat type – to represent a variety within the site e.g. woodland, semi-woodland, clear-cut, natural grassland, in order to maximise chances of trapping the three selected species. Field voles prefer ungrazed grassland as grasses are their only diet (e.g. bents, fescues, hair grass); bank voles prefer deciduous woodland with ground cover and feed mainly on leaves, berries and seeds; wood mice prefer woodland but are highly adaptable and can be found in most habitats except open hills (Gurnell and Flowerdew, 2006) Mammal Society, 2011) (Figure 3.5)

- Visual evidence of rodent presence, e.g. droppings, runs, grass clippings, burrow entrances (Figure 3.6)

- Accessibility by vehicle for transportation of traps and sampling equipment
- Landowner permission

Figure 3.5 Examples of variety of habitat for wild rodent trapping: (a) rough pasture grazed intermittently by sheep (Cumbria); (b) clear cut area in cultivated conifer forestry (Borders) (c) deciduous woodland strip adjacent to grazed fields (Pentlands)

a)  **b)**  

Figure 3.6 Visual evidence of rodent activity (field vole) demonstrated by the presence of droppings (a) and grass clippings (b). For field voles presence of *Juncus* spp. (c) or *Deschampsia* spp. (d) was frequently indicative of the likely presence of runs as these are favoured food items.

(a)  **(b)** 

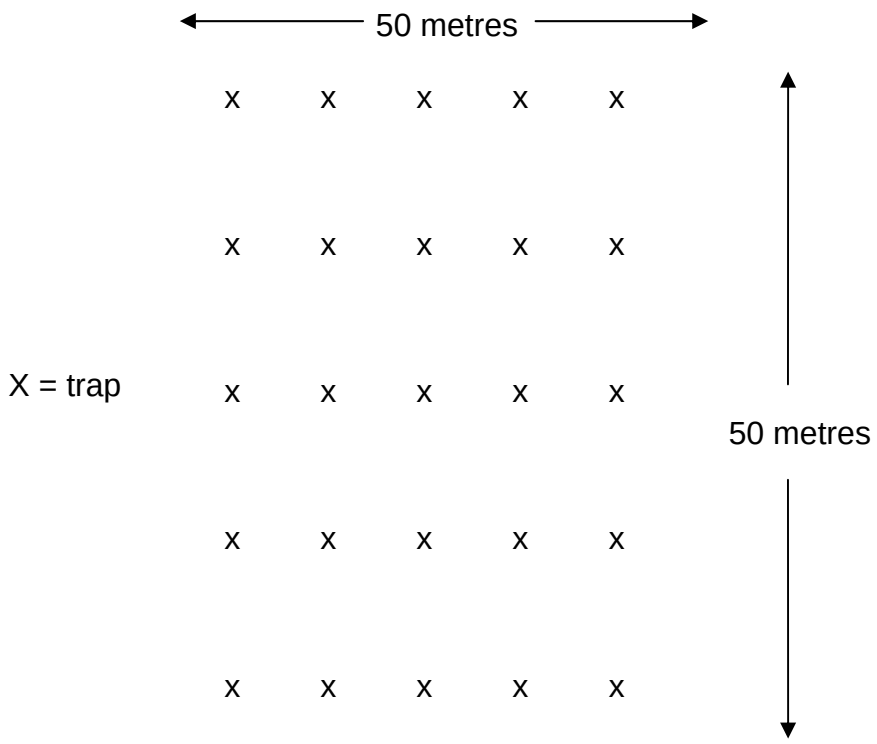
(c)  **(d)** 

Rodents were trapped using Ugglan No.2 traps with a storm roof (Grahnab, Sweden) (Figure 3.7). Trapping methodology was based on information from the Mammal Society (Gurnell and Flowerdew, 2006) and via direct training by personnel involved in an on-going study at Kielder Forest, Northumbria.

Figure 3.7. Ugglan trap with storm cover

Grids of 25 traps (5 x 5; approx. 10m spacing) were used. (Figure 3.8); traps were placed as close to the grid pattern as possible while still being in a suitable location, and therefore trap position could vary slightly from the rigid pattern. Trap position was marked with a bamboo cane

Figure 3.8 Schematic diagram of trapping grid



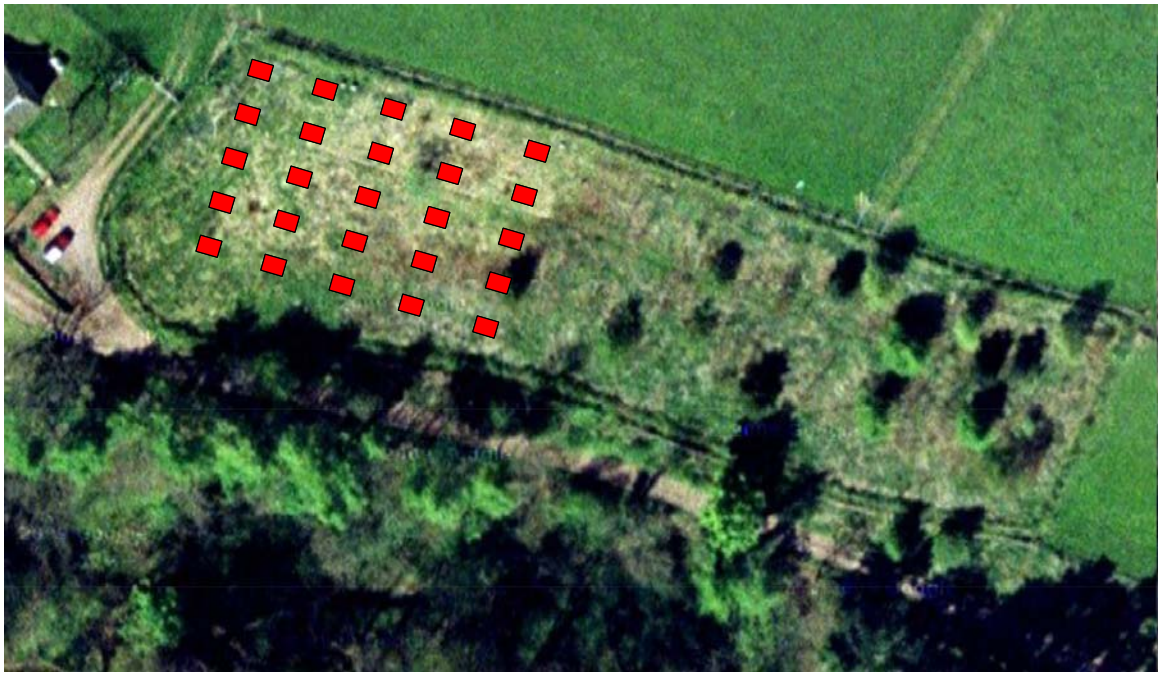
Traps were placed, prebaited with carrot and grain (Figure 3.9a and b) and left open for 3 days for habituation. The traps were then set in the evening of the 3rd, 4th and 5th days and checked the following morning. One trapping session therefore took 6 days.

Figure 3.9 Prebaited Ugglan trap (a) and in situ (b)

a) **b)**

An example study site (Pentlands) is shown in Figure 3.10 with a schematic representation of typical trapping grid layout.

Figure 3.10 Map of a typical study site with schematic representation of a trapping grid in place



Seasons

Trapping of wild rodents was divided into four seasons over a two year period, between April 2007 and December 2008 (Table 3.1). The Spring/Summer season covered the months April to early August and the Autumn/Winter season the months September to January.

Table 3.1 Summary of trapping seasons for wild rodents

Season	
1	Summer 2007 (April – August)
2	Winter 2007/8 (November – March)
3	Summer 2008 (April – August)
4	Winter 2008/9 (November – March)

Grid locations were varied within each site between the first and second year where possible. In the Pentlands and Borders study areas, 6 grids were placed within each area per trapping season. In the Cumbria study area, 12 grids were placed per trapping season, with the exception of season 4, when only 6 grids were placed. The increased number was due to the availability of a local assistant, who was trained to place and set traps.

Sampling

Trapped rodents were transferred into a plastic bag to avoid the stress of handling (Figure 3.11) and humanely killed on site immediately, by an overdose of volatile anaesthetic (isoflurane; IsoFlo™, Abbott Animal Health) followed by cervical dislocation. This is a Schedule 1 method of euthanasia under the Animals (Scientific Procedures) Act 1986 and thus does not require licence permission under the Act when performed by a skilled operator.

Figure 3.11 Once trapped, rodents were transferred without handling into a plastic bag.

A unique identifying number was allocated to each animal trapped and date of capture, grid location, trap location and species were recorded on site. Immediately after death, a cardiac blood sample was obtained using a 23 gauge needle and 2ml syringe, placed into a plain Eppendorf™ tube. A drop of blood was also placed on an FTA card (Whatman™, Whatman International Ltd). Samples and carcases were stored during trapping in a cooled freezer box. After each trapping session samples and carcases were removed to a laboratory within 2-4 hours where blood samples were centrifuged at 4000rpm to separate serum (approximately 200 µl in most cases) and blood pellet (S.Telfer, personal communication.).Each carcase was examined and the following data collected from each carcase: sex, age (juvenile, subadult, adult), reproductive status, bodyweight, body condition score (ranked scale 1-5), presence of ectoparasites, and presence of any external lesions. Each carcase was subjected to a post mortem examination (excluding brain). Any gross internal lesions were noted and samples of any such lesions taken and preserved in formal saline for future examination. Information obtained was recorded for future examination outwith this study.

Carcases, serum, blood pellet and kidney samples were then frozen at -70°C or in the Cumbria study area were stored at -20°C for 1-2 days before being transferred to -70°C.

3.3.2 Rabbits and Rabbit Haemorrhagic Disease Virus (RHDV)

Rabbits were obtained from the Borders study area by shooting as part of routine pest control. There was no control of rabbits in the Pentlands or Cumbria sites and it was found not possible to obtain rabbits from these areas. Only 16 rabbits were obtained from the Borders study area over the first year of the study. Due to this low number and the lack of samples from other study areas, it was decided after the first year to limit investigation of prey species to wild rodents only. As a consequence of this, serological testing for rabbit haemorrhagic disease virus (RHDV) was not undertaken as this virus is specific to rabbits and not known to occur in rodent species.

3.3.3 Foxes

Fox carcases were obtained opportunistically between January 2007 and January 2009 via routine fox control by shooting. For the Pentlands and Borders study areas, carcases were collected within 24 hours. For the Cumbria study area, carcases were stored locally at -20°C until collection. Each carcase was labelled with a unique identifying number and date and site of shooting (Ordnance Survey grid reference) recorded.

Post mortem examination was carried out immediately after carcase collection. Blood was collected from the thoracic or abdominal cavity and centrifuged at 4000rpm to remove solids. The supernatant was removed to a fresh Eppendorf™ tube. For carcases from the Cumbria study area, the local assistant removed blood or bloody body fluid as soon as possible after shooting and stored this separately to the carcase.

Fresh or defrosted fox carcases were subjected to a post mortem examination. Foxes were sexed and categorised as juvenile (milk teeth present), subadult (6-12 months) or adult (>12 months), based on body size and proportions, presence or absence of permanent dentition and a visual assessment of tooth wear. Permanent dentition is complete by 5-6 months, adult size is reached by 6-7 months and sexual maturity and ossification of the skeleton is reached at approximately 10 months in the fox (Harris, 1978) (Sullivan, 1956). (Backer et al., 2001) (Backer et al., 2001) Information

on reproductive status, body weight, body condition score (ranked scale 1-5), presence of ectoparasites and any external lesions was recorded. After internal examination kidney samples were removed. Any gross lesions were noted and appropriate samples taken and fixed in formal saline where indicated. Tissue samples were taken, including brain (n= 26) and pluck (heart and lungs) (n = 52), for submission to other collaborative studies and to archive for future study. Serum or body fluid and kidney samples were stored at -70°C. Whole fox carcases were not stored frozen after post mortem examination due to space constraints.

3.3.4 Cats

Serum samples were obtained from domestic cats via veterinary practices (Appendix 3) situated within the study area. Cat samples were collected opportunistically between January 2007 and May 2009 from animals presenting to the veterinary surgeon for blood sampling for veterinary diagnostic investigation. Cats were selected on the basis of a questionnaire completed by owners (Appendix 3) that confirmed that they had outdoor access and were known to hunt and consume rodents, rabbits or birds. Serum samples were acquired as surplus to that used for diagnostic investigation of cats presented to the veterinary practice, and thus obtained under the Veterinary Surgeon's Act 1966. Plain serum tubes and questionnaires were distributed to these practices and samples were stored at -20°C on the practice premises until collection. A letter explaining the purpose if the study and requesting informed signed owner consent was attached to the questionnaire and details regarding address, age, sex, indoor/outdoor status and hunting habits of each cat were obtained (Appendix 3). Although permission was obtained under the Animals (Scientific Procedures) Act 1986 to take a blood sample from cats for the primary purpose of the study, with informed owner consent, this approach was not used. Cat serum samples were stored at -20°C both after collection by the veterinary practice and prior to testing at the laboratory.

3.3.5 Corvids

Corvids were obtained via routine pest control practices at the Borders and Cumbria study areas only. Corvids were collected opportunistically between January 2007 and May 2009. In both areas, crows were trapped using a Larsen trap and killed by concussion of the brain by striking the cranium sharply, with death confirmed by

cervical dislocation. This is a schedule 1 technique under the Animals (Scientific Procedures) Act 1986 and thus does not require licence permission under the Act when performed by a skilled operator. Each bird was assigned a unique identifying number and the location of trapping recorded (Ordnance Survey grid reference).

In the Borders area, blood was obtained immediately after death by jugular venepuncture or by opening the body cavity and withdrawal of cardiac blood. In the Cumbria study area blood was obtained as soon as possible after death by the local assistant by opening of the body cavity, severance of the major vessels and aspiration using a 2ml syringe, before being placed in a plain blood tube.

Corvid serum or blood samples and carcases were stored at -20°C.

3.4 Results

Over the study period a total of 1096 animals were collected or sampled for further use in the study (See Table 3.2). These consisted of 905 rodent prey species (bank voles, wood mice, field voles), and 191 predator species (126 foxes, 28 cats, 37 corvids).

Table 3.2 Summary of prey and predator species collected from the three study sites

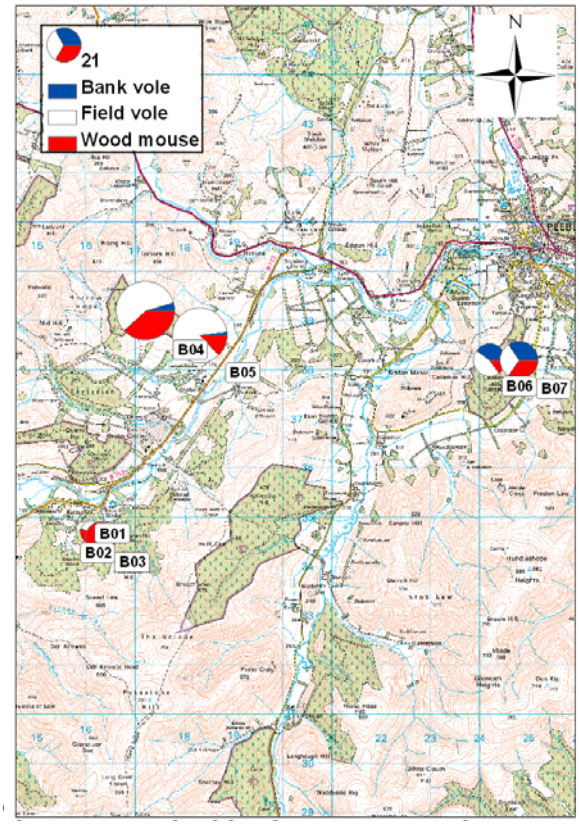
	Prey species				Predator species				
Area	Bank vole	Wood mouse	Field vole	Total prey	Fox	Cat	Corvid	Total predators	Total animals
Borders	22	64	107	193	52	11	18	81	274
Cumbria	76	118	124	318	50	9	19	78	396
Pentlands	116	165	113	394	24	8	0	32	426
TOTAL	**214**	**347**	**344**	**905**	**126**	**28**	**37**	**191**	**1096**

3.4.1 Prey species

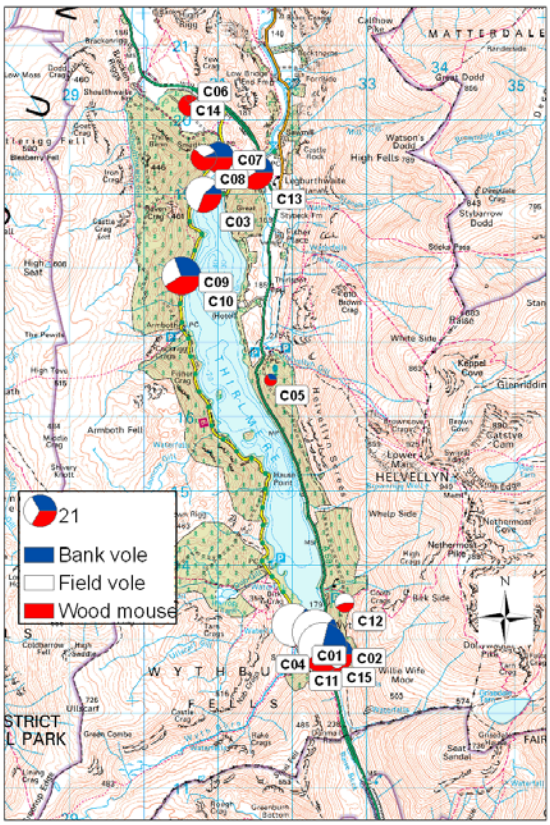
Maps indicating trapping site locations and numbers of rodents caught in each site by species are given in Figure 3.12. The Cumbria area had twice as many trapping sites (grids) in each season (12, as compared to 6 in the Pentlands and Borders areas) and an extra trapping season (season 4). Some grids were moved slightly from one season to the next, because access to the exact same location was not possible during that particular trapping period, and so overall the Borders had 7 separate grid locations, Cumbria 15 and Pentlands 6 (Figure 3.12).

Figure 3.12 Maps of the three study sites indicating trapping grid locations and pie charts giving total numbers of rodents caught by species

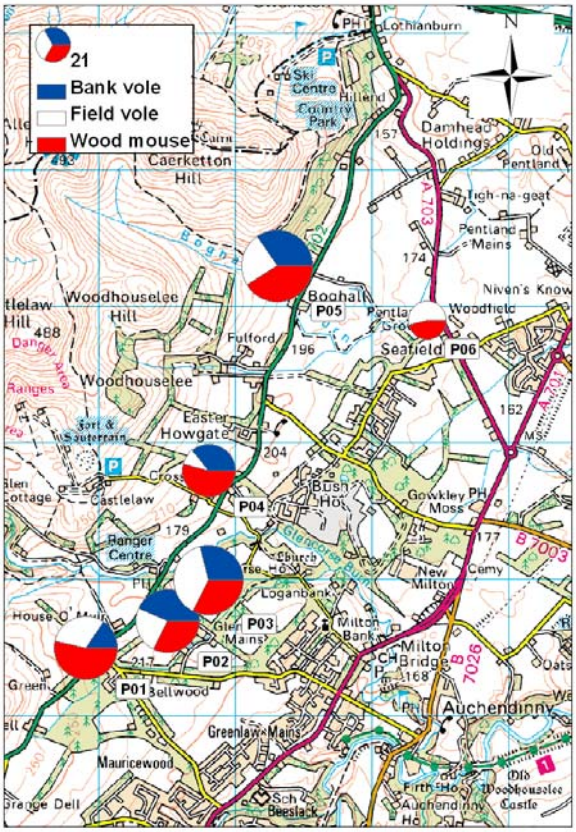
a) Borders (sites B01-B07)



b) Cumbria (sites C01-C15)



c) Pentlands (sites P01-P06)

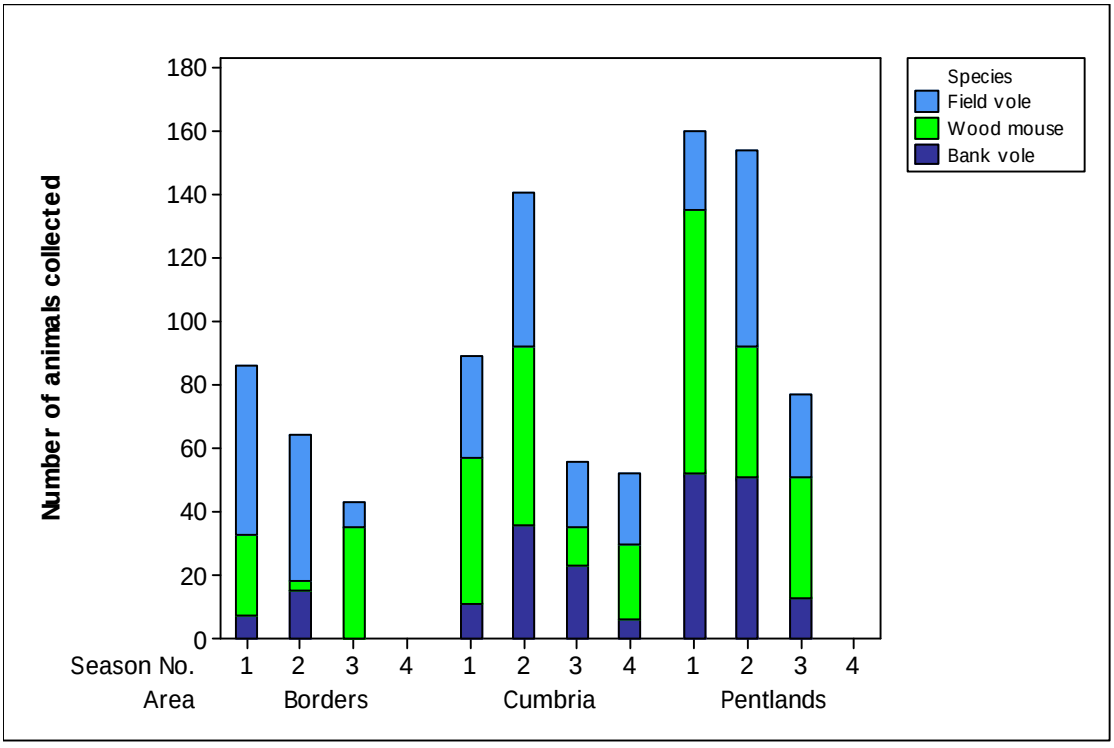


Numbers and species of prey species trapped and sampled in each of the four seasons are shown in Table 3.3 and Figure 3.13. In season 4 (Autumn/Winter 2008/9) trapping of rodents was only carried out at the Cumbria site. Rabbits were not investigated further in this study due to the low numbers obtained and lack of samples from the Cumbria and Pentlands study areas (see 3.3.2).

Table 3.3 Summary of prey species trapped per season and area

Area	Season no.	Bank vole	Wood mouse	Field vole	Total
Borders	1	7	26	53	86
	2	15	3	46	64
	3	0	35	8	43
	All	22	64	107	193
Cumbria	1	11	46	32	89
	2	36	56	49	141
	3	23	12	21	56
	4	6	4	22	32
	All	76	118	124	318
Pentlands	1	52	86	25	163
	2	51	41	62	154
	3	13	38	26	77
	All	116	165	114	394
	TOTAL	**214**	**347**	**344**	**905**

Figure 3.13 Barplot of the numbers of rodent prey species trapped per area and season



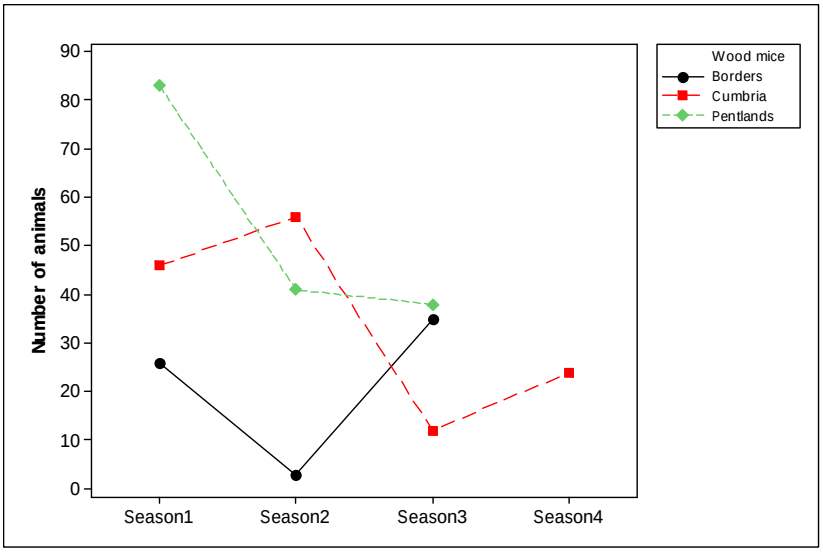
Overall, similar numbers of wood mice (347; 38%) and field voles (344; 38%) and fewer bank voles (214; 24%) were caught, but the predominant species varied depending on area, with field voles predominating in the Borders and Cumbria study areas (55% and 39% of the total number of rodents from that area respectively) and wood mice in the Pentlands (42%). Bank voles were the least numerous species caught in all three study sites.

There were seasonal variations in the numbers of rodents collected (Figure 3.13). In the Borders and Pentlands study areas, total numbers of prey species collected declined sequentially over the three trapping seasons. In Cumbria, total prey species collected were higher in season 2 compared to season 1, but subsequently declined over seasons 3 and 4. Annually, numbers of rodents trapped were lower in all study areas in the spring/summer of the second year of the study (season 3, 2008) compared to the first (season 1, 2007) declining by 50% in the Borders, 37% in Cumbria and 53% in the Pentlands. Prey were only trapped in the autumn/winter season over the two years in the Cumbria area (seasons 2 and 4), but in this area numbers were also lower in 2008/9 (seasons 1 and 2) compared to 2007/8 (seasons 3 and 4) with a decline of 77%.

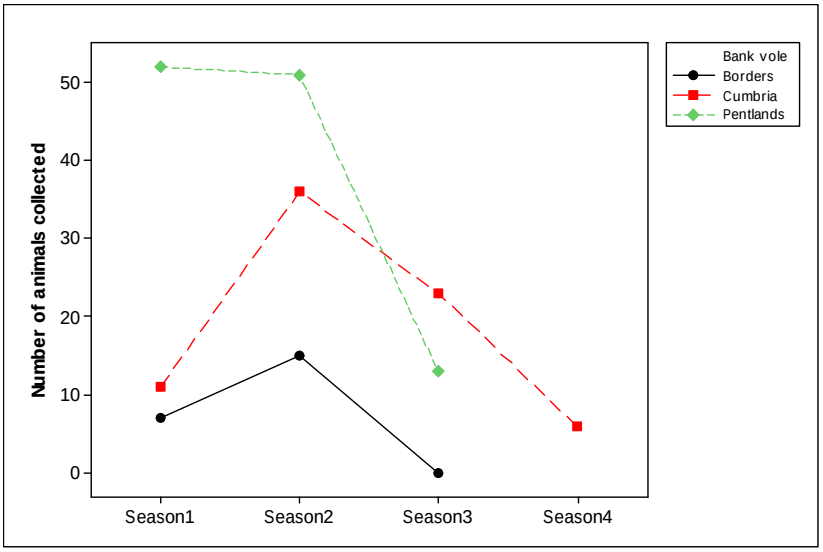
Although overall numbers of prey species were lower in the second year (season 3 compared to season 1), there were species differences (see Figure 3.14) and some species increased in numbers trapped in consecutive seasons. Only the Cumbria site shows a roughly similar pattern in numbers collected in the 4 seasons for all three species, with an increase in numbers trapped from seasons 1-2, followed by a decrease between seasons 2-3 and 3-4.

Figure 3.14 Seasonal variations in numbers of prey species collected from each study area: a) Wood mice b) Bank vole c) Field vole.

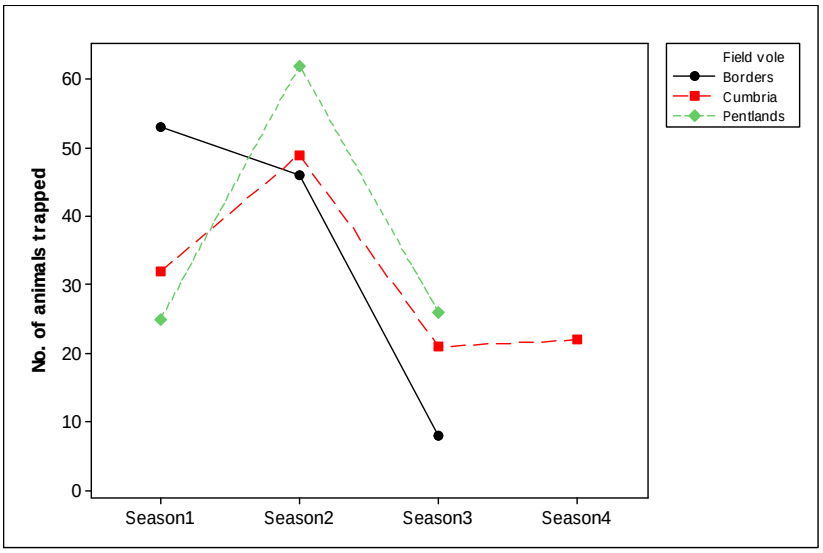
a)



b)



c)



Trapping success

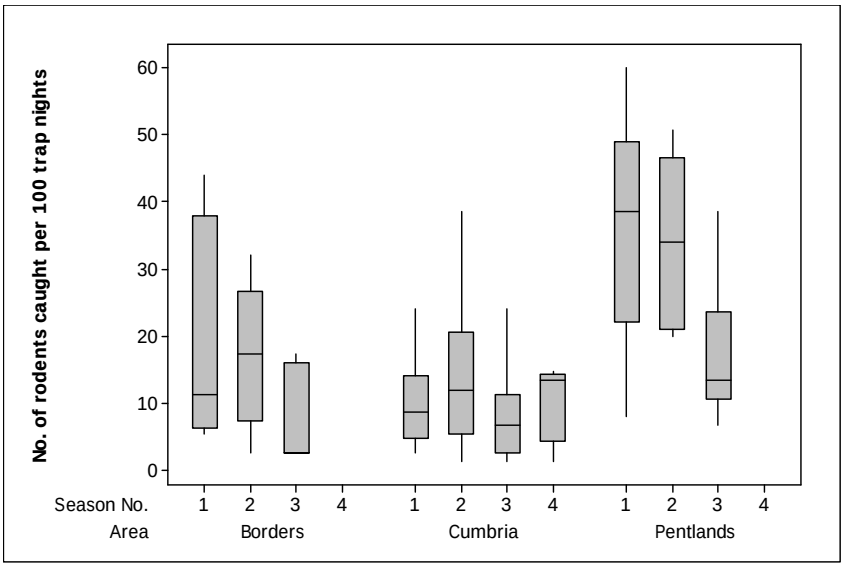
Trapping success, or index of abundance, is generally expressed as number of captures per 100 trap nights (tn) (Redpath et al., 1995). Trap nights are defined as number of traps multiplied by the number of nights for which the traps are set. Overall trapping success per season for each study area ranged from 6.2-36.2/100tn (Table 3.4), and success varied within areas, (e.g. 9.6-19.1/100tn for Cumbria) and by season (Figure 3.15)

Table 3.4 Trapping success (captures per 100 trap nights (tn)) for each study area per season.

Area	Season no	No. of traps	No. of nights	Total trap nights	No. rodents caught	Captures/ 100 tn
Borders	1	150	3	450	86	19.1
	2	150	3	450	64	14.2
	3	150	3	450	43	9.6
Cumbria	1	300	3	900	89	9.9
	2	300	3	900	141	15.7
	3	300	3	900	56	6.2
	4	150	3	450	32	7.1
Pentlands	1	150	3	450	163	36.2
	2	150	3	450	154	34.2
	3	150	3	450	77	17.1

Within each area, different sites had different trapping success and this varied seasonally (data not shown).

Figure 3.15 Boxplot of overall trapping success (number of rodent captures per 100 trap nights) per season for each study area. Horizontal line indicates median value, upper whisker extends to the maximum data point within 1.5 box heights from the top of the box, lower whisker extends to minimum data point within 1.5 times box heights from the bottom of the box.



Trapping success also varied for each of the three rodent species caught (Table 3.5), with bank voles ranging from 0 - 11.56, wood mice from 0.67 - 19.11 and field voles from 2.33 - 13.78.

Table 3.5 Trapping success (captures per 100 trap nights (tn)) for each rodent species per study area and season.

Area	Season no	Bank vole captures/100tn	Wood mouse captures/100tn	Field vole captures/100tn
Borders	1	1.55	5.78	11.78
	2	3.33	0.67	10.22
	3	0	7.78	1.78
Cumbria	1	2.44	10.22	7.11
	2	8	6.22	5.44
	3	2.56	1.33	2.33
	4	1.33	0.89	4.89
Pentlands	1	11.56	19.11	5.56
	2	11.33	9.11	13.78
	3	2.89	8.44	5.78

Age and sex distribution of prey

The majority of prey were adult (97.3%) and similar proportions of males (49.8%) and females (50.2%) were trapped (Table 3.6).

Table 3.6 Age and sex of prey species caught per area

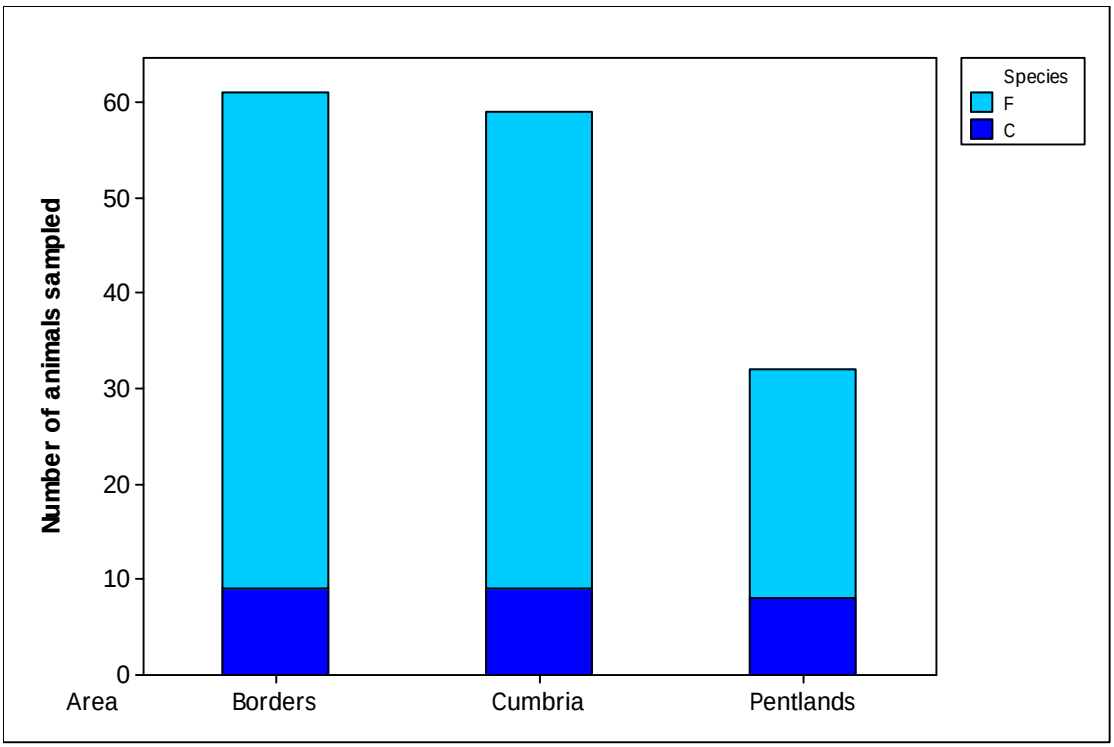
Area	Species	Adult	Juvenile	Female	Male
Borders	BV	22	0	7	15
	FV	107	0	55	52
	WM	60	4	24	40
	All	**189**	**4**	**86**	**107**
Cumbria	BV	76	0	34	42
	FV	122	2	84	40
	WM	117	1	55	63
	All	**315**	**3**	**173**	**145**
Pentlands	BV	114	2	49	67
	FV	105	8	72	41
	WM	158	7	74	91
	All	**377**	**17**	**195**	**199**
TOTAL		**881**	**24**	**454**	**451**

3.4.2 Predator species

A total of 191 predator species were sampled, consisting of 126 foxes, 28 cats, and 37 corvids (Table 3.2). Corvids were obtained from the Borders (n=18) and Cumbria (n=19) sites. Sample quality for both foxes and corvids was a problem at the Cumbria site, as blood was taken post mortem and already clotted, and a centrifuge was not available locally. However, due to the doubtful or non-applicability of the developed pathogen tests for avian blood (see Chapters 4, 5 and 6), corvids as sentinels were not evaluated further in this study.

The predator species on which pathogen testing was focused were therefore foxes and cats. Figure 3.16 presents the number of cats and foxes obtained from each study area. Similar numbers of cats were obtained from each area (11, 9 and 8 from Border, Cumbria and Pentlands respectively). Similar numbers of foxes were obtained from the Borders and Cumbria areas (52 and 50 respectively), and approximately half this number (24) were obtained from the Pentlands area.

Figure 3.16 Numbers of cats and foxes sampled from each study area

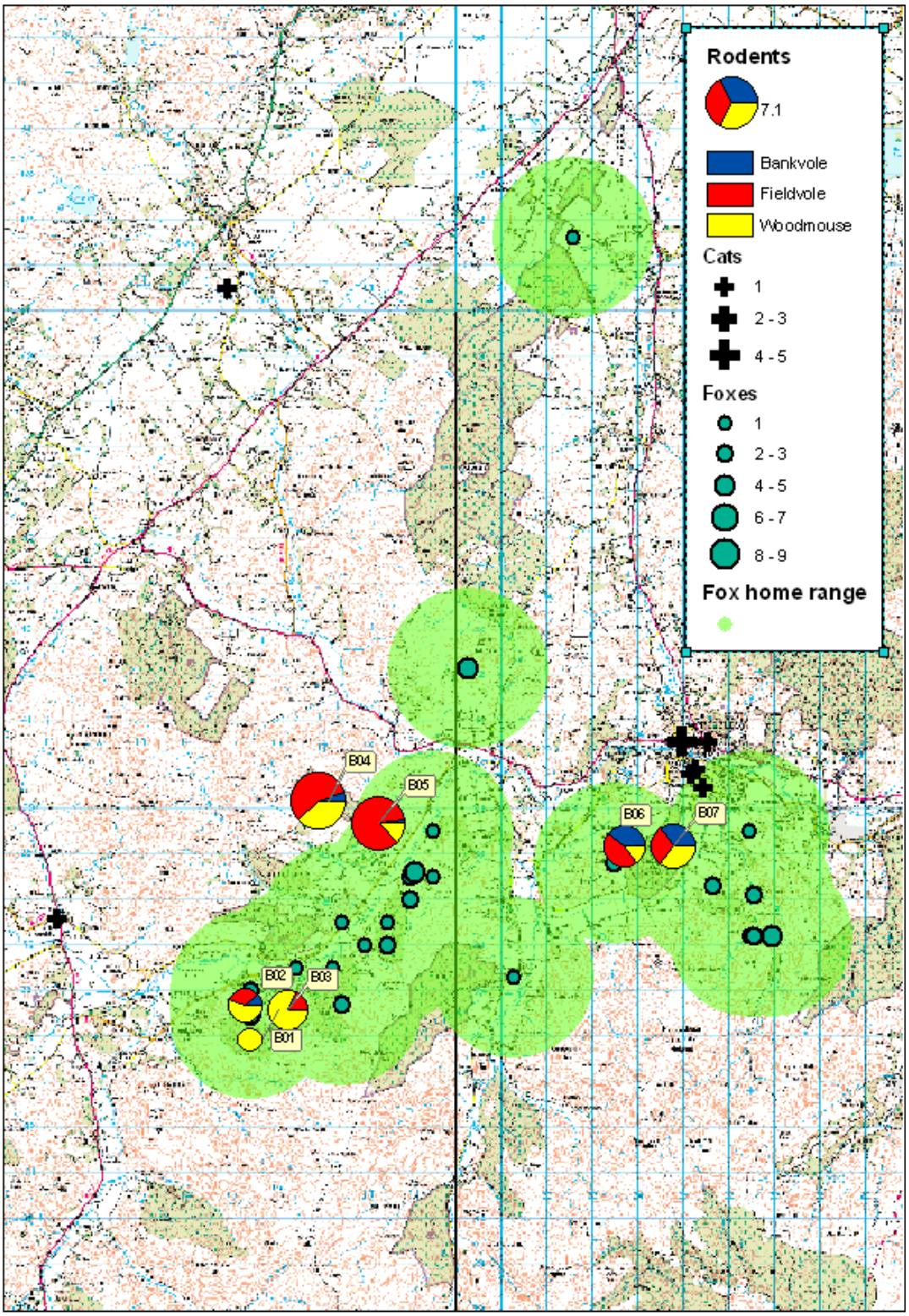


Maps of locations of foxes and cats, and relation to the rodent trapping sites are

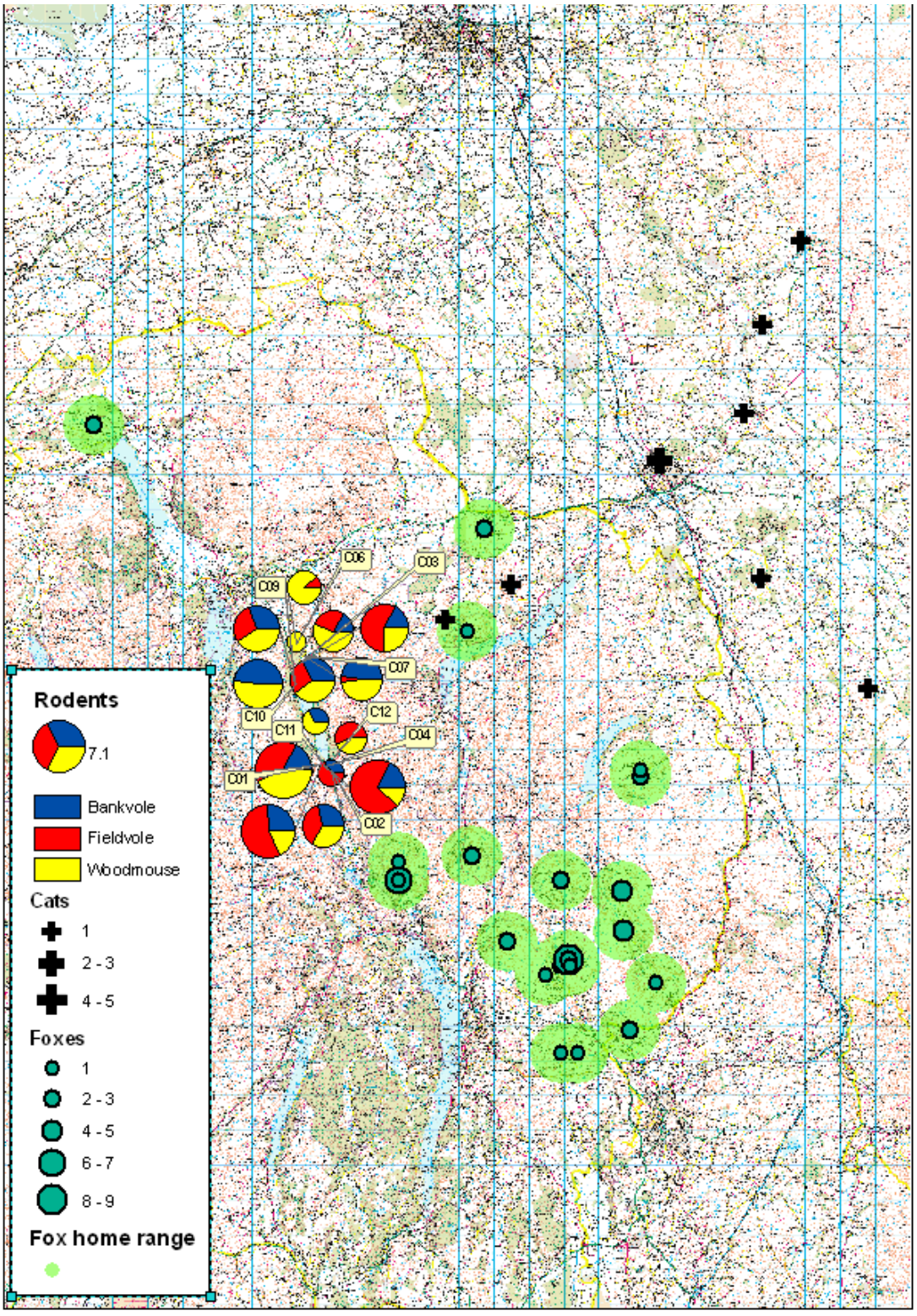
shown in Figure 3.17 for each study area.

Figure 3.17. Maps indicating locations and numbers of predators (foxes and cats) in relation to rodent trapping sites for a) Borders, b) Cumbria and c) Pentlands study areas. Fox home ranges are estimated at 10km^2

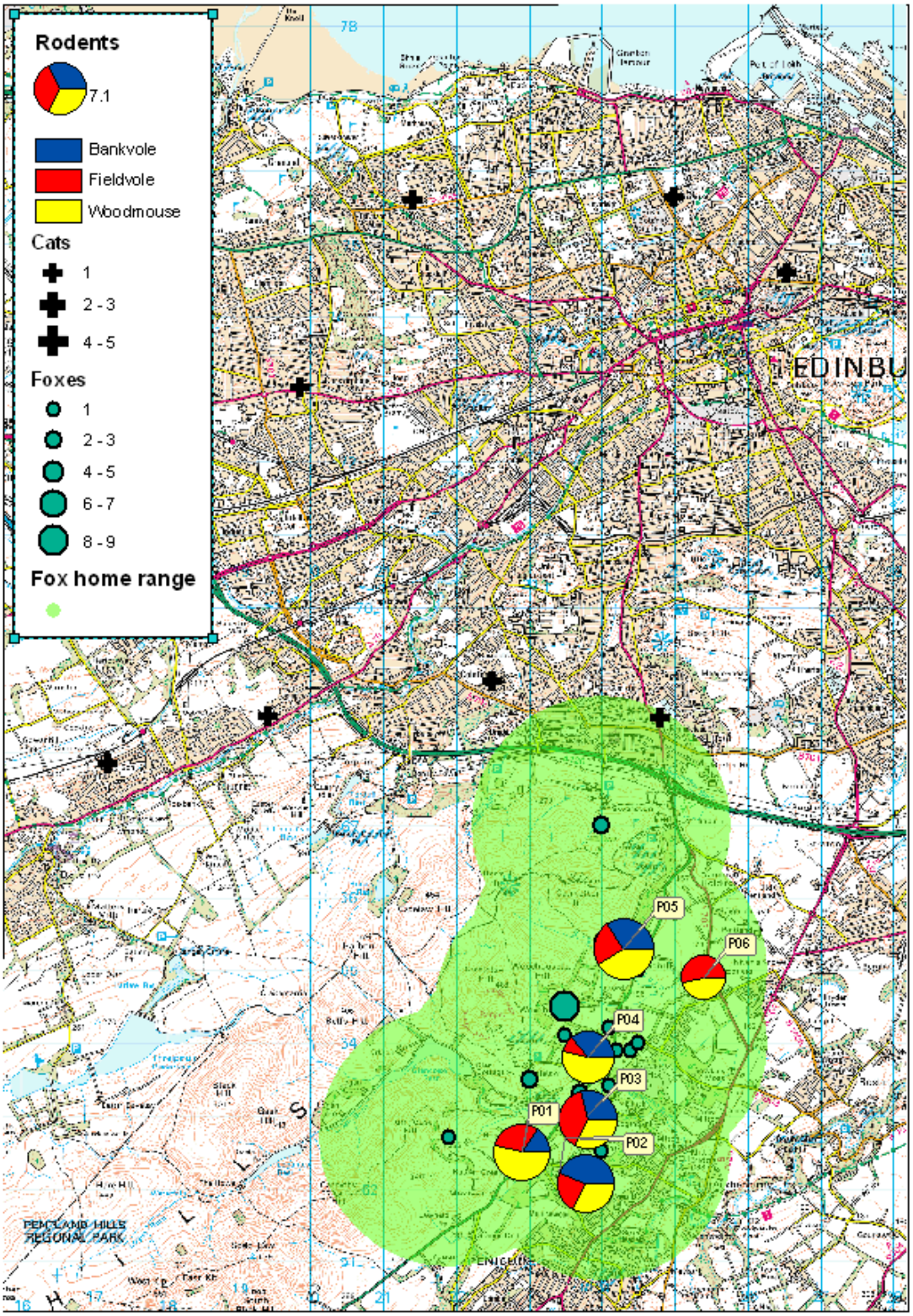
a) Borders



b) Cumbria


C06
C08
C09
C07
C10
C12
C11
C04
C01
C02
Rodents
7.1
Bankvole
Fieldvole
Woodmouse
Cats
1
2 - 3
4 - 5
Foxes
1
2 - 3
4 - 5
6 - 7
8 - 9
Fox home range

c) Pentlands

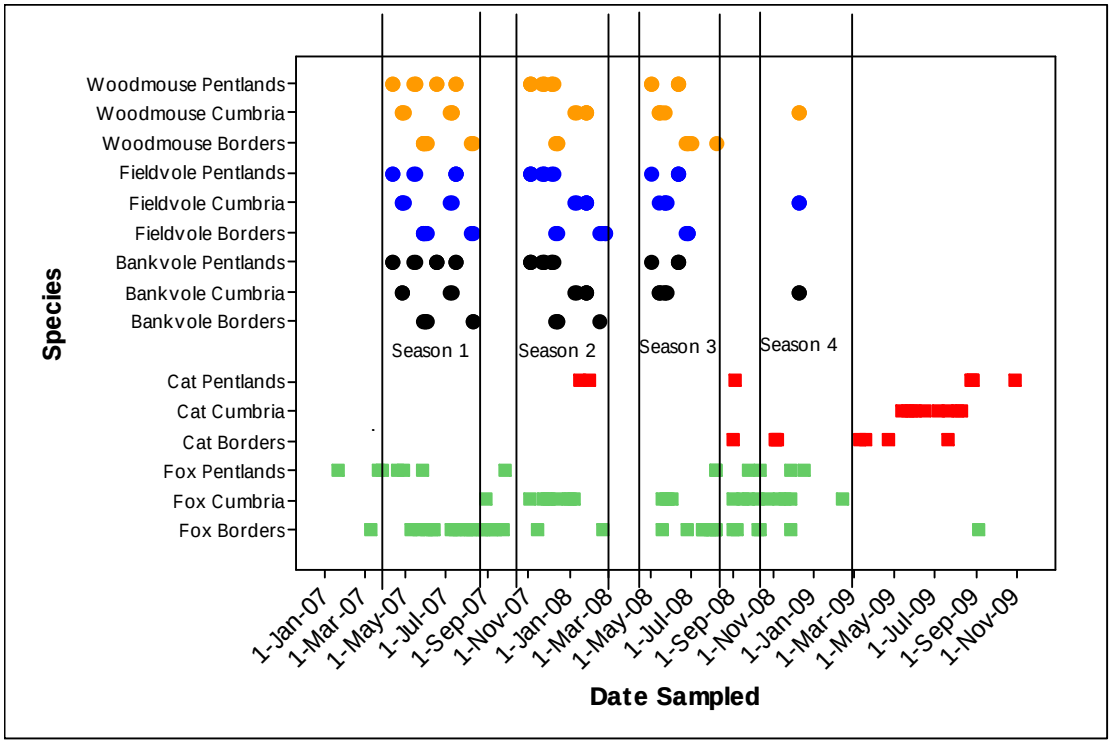

Rodents
7.1
Bankvole
Fieldvole
Woodmouse
Cats
1
2 - 3
4 - 5
Foxes
1
2 - 3
4 - 5
6 - 7
8 - 9
Fox home range
P05
P06
P04
P03
P01
P02
PENTLAND HILLS REGIONAL PARK
EDINBU

Study areas varied in the degree of overlap of estimated fox home ranges (10km^2) with the rodent trapping sites. In the Pentlands (Fig 3.17c), all the trapping sites were within the home ranges of sampled foxes, and in the Borders (Fig 3.17a) 6 out of 7 trapping sites were within the home range of sampled foxes. However in Cumbria none of the trapping sites were within the estimated home range of any of the sampled foxes (Fig 3.17b). For cats, home ranges were not mapped as they are much smaller than that of foxes - estimated at less than 1 ha for domestic suburban cats (Bradshaw, 1992), and none of those sampled overlapped directly with any of the trapping sites in any area. In the Pentlands area, all the cats came from a more urban environment than the rodent tapping sites (Fig 3.17c).

3.4.3 Sampling timeline of prey and predators

Foxes were obtained throughout every trapping season, and in the periods before, between and after each trapping season (except between seasons 2 and 3) for the duration of the study (Figure 3.18). Cats, however, were only obtained during seasons 2 and 4, between seasons 3 and 4, with the majority after season 4 and when trapping of rodents had finished. For the purposes of analysis, foxes were allocated to the corresponding rodent season by placing a cut off at the midpoint between one rodent season and the next. Cats were not allocated a season.

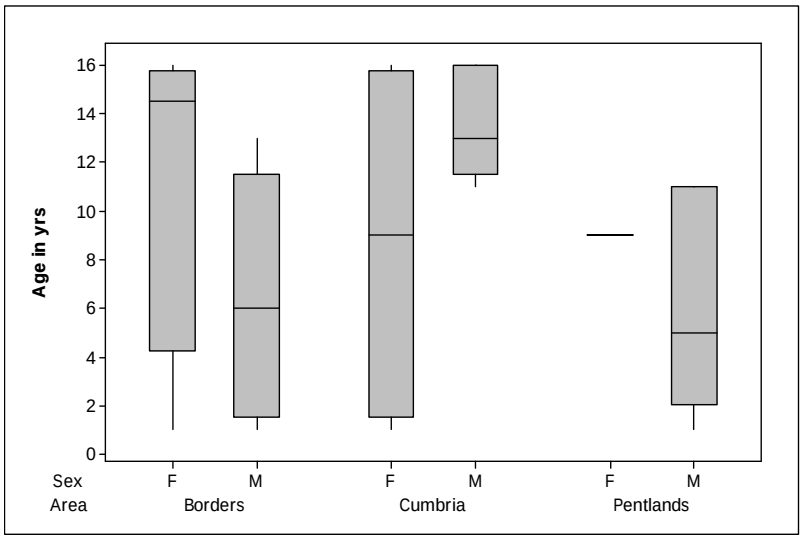
Figure 3.18 Timeline of when prey and predator species were sampled during the study period from January 2007 to December 2009. Trapping periods for prey species are indicated (seasons 1-4).



3.4.4 Age and sex distribution of predators

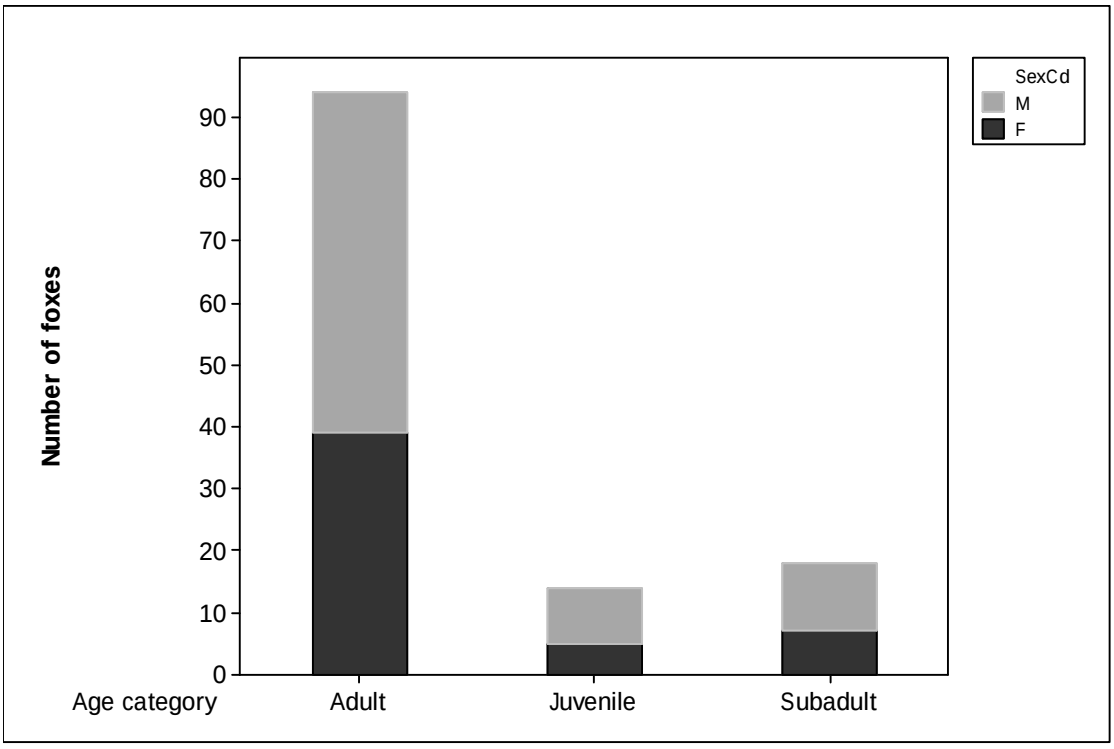
Age (in years) and sex details were obtained for 26 out of the 28 cats (Figure 3.19). Sixteen (62%) were greater than 9 years old. 9/26 (35%) were female and 17/26 (65%) were male. Of the 26 cats of known age, 24 (92%) were alive during the entire study period and all four trapping seasons.

Figure 3.19 Box plot of age and sex of cats in each study area (as for Figure 3.15)



For foxes, forty percent (n= 51) were female and 60% (n=75) were male. The majority were adults (74.6 %; n=94), 14.3% (n=18) were subadults and 11.1% (n=14) were juveniles. Age category and sex distribution are shown in Figure 3.20, and numbers in each age category per area are given in Table 3. 8. Foxes from all three age categories were sampled in the Borders area, while only adults were obtained from the Cumbria area.

Figure 3.20 Age category and sex distribution of foxes sampled (n=126)



3.4.5 Samples submitted for serological testing

Serum samples from both prey and carnivore species were tested for the presence of antibodies to the three remaining selected pathogens – *C. burnetii*, *Leptospira spp.* and *E.cuniculi*. Of the 1177 samples initially collected, 118 were not used further in this study (16 rabbit, 37 corvid, 65 shrew). Tests for RHDV were not performed as insufficient rabbit samples were collected during the study period (see 3.3.2). Corvid samples were not tested due to applicability of the serological tests (ELISA) and conjugates used (see Chapters 4, and 5).Of the remaining 1059 samples, 924 (87.2 %) were tested for antibodies to *C. burnetii*, 952 (89.9%) to *Leptospira* spp. and 921

(86.9%) to *E.cuniculi*. Numbers and species of serum samples tested for each pathogen are summarised in Table 3.7. For some samples, insufficient serum was available to test for one or more pathogens. Commercially available tests were used and adapted, or, where necessary, new tests were developed. These and the results obtained are described in chapters 4, 5 and 6.

Table 3.7 Numbers and species of serum samples tested for *C. burnetii*, *Leptospira* spp and *E.cuniculi* from each study area

Area	Species	Prey Predator	No. tested *C.burnetii*	No. tested *Leptospira*	No. tested *E.cuniculi*
Borders	Cat	Predator	11	10	11
	Fox	Predator	39	40	37
		Total predator	**50**	**50**	**48**
	Bank vole	Prey	17	19	19
	Field vole	Prey	99	99	101
	Wood mouse	Prey	55	57	56
		Total prey	**171**	**175**	**176**
Cumbria	Cat	Predator	9	9	9
	Fox	Predator	46	48	47
		Total predator	**55**	**57**	**56**
	Bank vole	Prey	61	64	60
	Field vole	Prey	113	115	109
	Wood mouse	Prey	99	104	97
		Total prey	**273**	**283**	**266**
Pentlands	Cat	Predator	6	7	7
	Fox	Predator	17	18	17
		Total predator	**23**	**25**	**24**
	Bank vole	Prey	102	106	99
	Field vole	Prey	97	101	102
	Wood mouse	Prey	153	155	150
		Total prey	**352**	**362**	**351**
TOTAL			**924**	**952**	**921**

3.5 Discussion

In order to collect serum samples for pathogen testing from predators and prey in pursuit of the proof of principle for this study, appropriate methods of obtaining the targeted species from each selected study area had to be employed. Compared to ecological studies aimed at the prey population, for this study the key issues were ease of sampling, acquisition of sufficient numbers, and how representative the animals sampled are with respect to exposure to the pathogen under investigation in that area.

Evaluation of sample collection methodology

Prey

For wild rodents, targeted trapping is the only feasible means of obtaining serum samples. Trapping methods for small rodents vary widely in terms of trap type and layout, and depend on the purpose of the study. For example, unlike in this study, trapping is frequently used to estimate population size or density, and various methods such as capture-mark-recapture, and differing mathematical models and computer software are widely described in the literature (Menkens, Jr. and Anderson, 1988) (Nichols and Pollock, 1983); http://warnercnr.colostate.edu/~gwhite/software. html).

Trapping success can be used as an estimate of abundance and is generally expressed as number of individuals captured per 100 trap nights, also referred to as index of abundance (Flowerdew, 1976; Gurnell and Flowerdew, 2006). Spring traps were not used in this study, but where they are used success is expressed as numbers per 100 corrected trap nights (100 ctn $^{-1}$) which accounts for the unavailability of traps that have been sprung but have not caught an animal (Nelson and Clark, 1973). Many studies have examined the effects of trap type (Anthony et al., 2005; Innes and Bendall, 1988; Jacob et al., 2002; Lambin and MacKinnon, 1997), trapping period (Olsen, 1975), trap bait (Chitty and Kempson, 1949; Gurnell, 1976), trap spacing (Tew et al., 1994) and trap position (Gurnell and Langbein, 1983) on small mammal trapping success. Many commercial live trap types are available, e.g. Longworth®, Sherman®, Wellfield Small Mammal traps, and trap type and size can influence species caught and affect mortality rates (Anthony et al., 2005). Traps may be laid in various patterns such as grids, transects, or webs, and differing baits and covers used (Anderson et al., 1983; Pearson and Ruggiero, 2003; Weihong et al., 1999). In this study, although trapping success (animals caught per 100 trap nights) could be used as a crude index or estimate of prey abundance in each trapping site (Redpath et al., 1995), this method assumes that this measure of relative abundance is directly related to absolute abundance; however it has been shown that non-linear relationships can be obtained (Tanaka, 1960), especially at densities above 20 capture/100tn. Determination of absolute density of rodent population has been described by Zippin

(1958) using plots of nightly catch against cumulative catch to estimate the number of rodents left untrapped in a study area by extrapolation (Zippin, 1958), but other studies have shown this method to be unreliable for some rodent species (Brown et al., 1996) Telfer S, personal communication).

Various other factors can also affect trapping success such as trap odour (Gurnell and Little, 1992), and it has been demonstrated that voles enter dirty traps significantly more than clean traps (Boonstra and Krebs, 1976). In this study traps were not washed between trap nights, aiming to minimise any possible deterrent effect of a clean trap. Traps were power-washed with water at the end of each period of use (6 days) in each grid, so were clean when initially placed, but the three days pre-baiting allowed movement of animals freely through the traps for feeding and deposition of faeces and urine so when traps were set they were likely to have rodent odour.

Although live trapping, blood sampling and release is possible without killing the animal, only small quantities of blood can be obtained (typically 20-30 µl) (Burthe et al., 2008) . The large quantity of serum required and the possibility of using other body tissues for collaborative or future studies of pathogen detection, led to the decision to euthanase the rodents once caught. Snap traps, which kill the animal, were not deemed suitable as a fresh non-clotted blood sample was required.

The methodology for this study was based on that used successfully by the National Centre for Zoonosis Research (NCZR) at the University of Liverpool for ongoing studies into the ecology of natural infections in wild rodents (S.Telfer, personal communication) and practical field training in habitat selection, trap location and rodent handling and processing was provided by NCZR. The Ugglan trap is a cheaper alternative to the Longworth trap and has been shown to be as efficient as the Longworth in terms of numbers of animals caught (Lambin and MacKinnon, 1997). This study was not primarily concerned with detailed population density estimates for the rodent species caught, but trapping success is a good general indicator of abundance and this parameter is often used to follow population fluctuations (Korpimaki et al., 2005). The trapping success results obtained in this

study (6.1-36.2/100tn) are comparable with other studies, for example an overall trapping success for all three species of 10.68/100tn in Kielder Forest, Northumberland (Lambin et al., 2000), 10.34/100tn (%) for bank voles in France (Augot et al., 2008), 0.2 – 8/100tn in Scottish moorland (Leckie et al., 1998) and 5.3 rats/100thn and 0.2 mice/100tn in New Zealand (Weihong et al., 1999).

One of the criteria used in selection of study areas and sites was to represent differences in rural geography and habitat type. Higher numbers of field voles were caught in both the Borders and Cumbria sites which is consistent with these sites having a predominance of sites of clear-cut areas of commercial forest and rough grassland, in which habitat this species has been shown to predominate (Lambin et al., 2000). Traps were also generally placed where there were field vole indices such as runs, droppings or grass-clippings so this species was partially targeted.

The Pentlands area was more intensively farmed with sites placed mainly at the edges of livestock fields or in natural mixed wooded areas, between livestock fields, and the predominant species caught was wood mice (42%). Wood mice live in underground burrows and commonly inhabit mainly woodland and fields but are very adaptable and can be found in most habitats (Mammal Society, 2011). Bank voles represented the smallest proportion of rodents caught in all three sites and this species tends to inhabit deciduous woodland and hedgerows and with good ground cover; this habitat was only available in a few parts of some sites in all areas. They have been shown to have increased abundance in old-growth moist forests (Olsson et al., 2005)**,** but this specific habitat was not available in any of the study areas.

Trapping success varied seasonally and annually in all areas with a sequential reduction in success over the three seasons in the Borders and Pentlands sites. Wild rodent populations are known to undergo regular annual cycles in abundance with increasing numbers over the summer and autumn, and decreased numbers in spring (Crawley, 1970; Montgomery, 1989).These fluctuations are believed to occur due to direct (social and territorial effects) and indirect (food supply, predation) density dependence (Krebs et al., 1973; Saitoh et al., 1999). Populations can also undergo

multi-annual natural cyclical variations in population size and the mechanisms underlying these variations are the subject of much scientific interest and controversy, with food supply ("bottom –up") and predation ("top-down") being the most widely proposed primary factors, but natural dispersal, cyclic weather patterns, disease and stochasiticity have also been suggested as potential causes (Korpimaki et al., 2005; Lambin et al., 2000; Wang et al., 2009). There may also be different primary factors in different regions (Lambin et al., 2006).

The normal annual pattern of abundance is reflected by the reduction in overall trapping success between season 1 and 2 (summer and winter) in both the Borders and Pentlands sites. However, this was not seen in Cumbria in the first year, with overall success here increasing from 9.9/100tn to 15.7/100tn. Within the Cumbria area, 10 sites were placed in the exact same location in both seasons 1 and 2. Six of these showed an increase in numbers caught between seasons 1 and 2, one yielded the same numbers in each season, and three sites had a decrease. Three of the 12 sites used in season 2 were in new locations so no direct comparison can be made. One of these new sites had a trapping efficiency of 100% (25 animals from 25 traps) in season 2. In the second year of the study, although the Cumbria area did have a drop in total numbers caught between season 3 and 4, only 6 grids were placed in season 4 compared to 12 grids in season 3 so the overall trapping success in fact increased between summer and winter from 6.2/100tn to 7.1/100tn. Of the 4 Cumbria sites that were in the same location in season 4 as in season 3, 3 yielded an increase and one yielded the same number between season 3 and 4. Possible reasons for this difference in seasonal pattern in the Cumbria site include the influence of factors that could override the normal density dependence of annual population abundance, such as good weather or an overabundant food supply, but this was not assessed directly.

There was a marked annual drop in trapping success between Summer 2007 (Season 1) and Summer 2008 (Season 3) in all three sites of 50% (Borders), 37% (Cumbria) and 53% (Pentlands). This suggests that the effects on the population by the removal of animals through trapping and killing could have made a significant impact, i.e. trapping mimicked intensive local predation and natural dispersal of animals from

adjacent areas into the trapping sites combined with presence and reproduction of animals not caught during the trapping period was insufficient to return abundance to the previous years' level. Alternatively there may have been similar or individual natural factors influencing populations in each study area such as adverse weather conditions and poor food supply, or 2007 was naturally a year of high abundance at the peak of the cycle and would have declined anyway in 2008 regardless of the effects of trapping.

Ninety seven per cent of all rodents trapped were adult, with similar proportions of males and females, even though males have been reported as being trapped more easily as they encounter more traps in their larger home range (Flowerdew, 1985). Other wild rodent studies have indicated that males are more commonly infected with pathogens (Hazel et al., 2000a; Telfer et al., 2007), therefore seroprevalence might be underestimated by sampling similar numbers of males and females. However, while it could be assumed that adult animals are more likely to have encountered a pathogen, due to increased numbers of encounters with conspecifics or environmental sources, there has been no clear association found between prevalence of infection and age (Hazel et al., 2000; Telfer et al., 2007).

Predators

Foxes

Acquisition of predator samples relied on submission from third parties. For foxes, this was relatively simple once local contacts in the three areas were made, principally with game-keepers. In the Borders and Pentlands, when a fox was shot a telephone call was made to arrange collection of the carcase for processing. In the Cumbria area, because this was remote from the research base, foxes were delivered by vehicle to the local assistant in Thirlmere, who sampled them and then stored the carcases in a -20C freezer until they were collected when the Cumbria site was visited for rodent trapping sessions. In the Borders and Pentlands sites, foxes were obtained from close to the rodent trapping sites (Figure 3.17a and c) and there was theoretical overlap between some estimates of rural home range of a fox in the UK of 600 hectares ($6km^2$) (Lloyd, 1980), to more than 1000 hectares ($10km^2$) in the

northern fells of the UK (Figure 3.17). Other studies from the UK and the rest of Europe and the USA give fox home ranges of 0.45km^2 to 9km^2 (summarised in (Trewhella et al., 1988), and in some studies up to over 5000 ha (50km^2) (Voigt and Macdonald, 1984).

It is known that home range has an inverse relationship with population density (Trewhella et al., 1988) and is affected by habitat richness (Lucherini and Lovari, 1996), but any specific information or studies on fox populations in the specific study areas used in this project that might give an indication of fox densities or home ranges were not available. However, it was never the intention of this study that predators needed be obtained from exactly the same areas as the prey animals, i.e. that there needed to be any certainty that an individual predator would have actually been likely to have consumed a prey item from the exact prey population sampled in each particular study site: rather, both the prey and predator populations should be representative of those in each study area and reflect any likely pathogen prevalence in these areas. This assumption would of course not be able to account for any highly localised pockets of infection with a particular selected pathogen, but again this was not the purpose of the proof of principle being tested in this study. In the Cumbria area (Figure 3.17b), it was not possible to get foxes from areas very close to the rodent trapping sites, and they were collected from up to 23km away from the rodent trapping sites, compared to a maximum of 4.2km in the Borders and 1.7km in the Pentlands sites. (See Chapter 7 for further discussion on home ranges and overlap of foxes and prey).

Sampling of fox carcases that were at least 12 hours post mortem, rather than fresh carcases immediately after euthanasia, greatly affected quality of blood and serum samples due to clotting, haemolysis and bacterial contamination. In many cases blood was collected from the thoracic cavity where it was mixed with contused lung tissue and pleural fluid from the fatal ballistic injury, and in many cases was contaminated with abdominal contents also. Despite centrifugation and filtering in some cases, quality of serum obtained from the foxes was always poor compared to that from the prey rodent species. Ideally, foxes should have been sampled

immediately after death, but training of fox control personnel and distribution of equipment to achieve this was not undertaken in this study. However, it should be considered for future studies in order to prevent sample quality issues.

Cats

There was considerable difficulty in getting compliance from selected veterinary practices to collect and submit cat serum samples for the study. Despite initial enthusiasm and agreement to contribute samples, it was found that, without regular reminders and personal visits, veterinary practices tended to omit to collect samples. This resulted in a delay in starting to obtain serum until towards the end of season 2 in the Pentlands, between seasons 3 and 4 in the Borders and after season 4 had ended in Cumbria. Numbers obtained were also disappointing, despite initial assurances from the veterinary practices involved in each site when first approached to participate that they would easily be able to obtain large numbers for the study. With hindsight, it is likely that it would have been more efficient to utilise the Home Office Project License authority to actively target and sample known house and farm cats in and close to the specific study areas by direct house visits etc., in order to ensure good numbers of cat samples. However, time and resource constraints prevented this approach in the current study (see Chapter 8 for further discussion). Cat serum samples were of good quality with no evidence of haemolysis or contamination. Storage in a freezer in the veterinary practice until collection occurred without a problem.

Due to collection of serum via veterinary practices cat samples are largely from nearby urban or suburban areas (Figure 3.17) in which the practices were located in most cases. Domestic cat (*Felis catus*) home ranges have been studied, but usually where this species is feral or semi-feral (free-ranging) and are known to vary about 200-fold depending on habitat, from 0.84 hectares (0.0084km^2) to 112 hectares (1.12km^2) (Fitzgerald and Karl, 1986; Liberg, 1980; Macdonald and Apps, 1978; Turner and Mertens, 1986; Warner, 1985). Suburban cat home ranges have been shown to range from less than 1 hectare to 27.93 ha (Barratt, 1997). Studies of cat behaviour have shown that home ranges of neighbouring cats frequently overlap,

with co-use of pathways and hunting grounds, but at different times (Leyhausen, 1965). Due to these much smaller home ranges compared to foxes, none of the cats sampled had estimated ranges that overlapped any of the study sites in any of the three areas. However, as for the foxes, it was not the intention of the study that this would be the case, but that they would be representative predators from that area and likely to reflect the area's selected pathogen prevalence.

Taking into account the timeline of predator and prey sampling (Fig 3.18) only the foxes were sampled concurrently with the prey trapping seasons and thus it could be argued they are more likely to accurately reflect levels of infection with a selected pathogen in the prey at the time of sampling.

Predator age distribution

Adult foxes could only be categorised as greater than 1 year old, and those sampled were unlikely to be much older than this from observed degree of dental wear, although more accurate estimates of ageing were not employed, such as counting dentine layers layers on longitudinally sectioned canine teeth (Roulichova and Andera, 2007). Lifespan in the wild is usually 1-2 years, although they have been recorded at up to nine years of age (Mammal Society, 2011), and thus the foxes sampled were likely to accurately reflect pathogen prevalence in the sampled prey as they were unlikely to have been alive much before the first period of trapping commenced (Figure 3.18). In contrast, rodent prey species generally live for less than 1 year (Mammal Society, 2011).

Age of predator is likely to influence degree of exposure to pathogens, i.e. with greater age and more predators consumed, the likelihood of exposure will increase, or there may be a cumulative effect whereby repeated or continued exposure and antigenic stimulation over time leads to a greater likelihood of seropositivity.

For human infectious diseases where infection and recovery induces lifelong immunity, many serological studies have shown that the proportion of a population seropositive to an infectious agent (e.g. measles, rubella, mumps, polio) rises steadily

with age, and the rate of rise directly reflects the force of infection (per capita rate at which susceptible individuals acquire infection) in that population (Anderson R.M. and May, 1992b). In domestic dogs, increasing age has been shown to be significantly associated with seropositivity to *Leptospira* spp. (Stokes et al., 2007; Ward et al., 2002). There are few studies investigating age seroprevalence in wild carnivores, but in Iberian Lynx seroprevalence to *Toxoplasma gondii,* believed to be largely from consumption of infected rabbits, has been shown to significantly increase with age (Garcia-Bocanegra et al., 2010), and in the Rocky Mountain cougar, age was the most important predictor of risk of exposure to various feline pathogens, including *Yersinia pestis,* which is maintained in rodent populations (Biek et al., 2006).

In contrast to the foxes, the cats sampled were alive when sampled, and 92% were alive during the entire study period and all four trapping seasons. Their mean age was 8.67 years (95% CI 3.87, 13.47) years in the Borders, 11.44 years (95% CI 7.07, 15.81) in Cumbria and 6.38 years (95% CI 2.75, 10.01) in Pentlands. These animals could thus potentially reflect selected pathogen prevalence not only during but also prior to the study period, even though the majority were not sampled until after prey trapping had finished. This would depend on the immune response to exposure to a particular pathogen being prolonged. Knowledge of the duration of seropositivity can be useful in certain situations; for example, foxes exposed to RHDV by ingestion of infected rabbits exhibit an immune response that only lasts a few weeks ((Frolich et al., 1998) and so seropositivity reflects only very recent exposure. In contrast, some animals that are long lived, develop a persistent response to exposure to a pathogen and can be reliably aged have the attributes to be used as 'retrospective' sentinels, as discussed by Halliday et al (2007), especially if they remain alive and are sampled sequentially over time.

There is also the possibility of cats moving location with their owners. Their seroprevalance may therefore reflect exposure to different prey populations in very different locations in the past, if antibody titres against the pathogens of interest are persistent for long periods of time after exposure. Information on any previous home

locations was not obtained for the cats, but with hindsight may have been useful. Overall, it would seem likely that foxes may be more useful as sentinels for the immediate or recent situation of pathogen prevalence in their prey, i.e if the question is, "is the selected pathogen present in this area now?", and have the potential to reflect pathogen presence from a larger area, whereas cats may not be able to answer this question as clearly due to their longevity and much more restricted home range.

The relationships between seroprevalence in the prey and predators sampled, as described in this chapter, and the effects of factors such as study area, season and species are now explored further for each of the selected pathogens, *Coxiella burnetii*, *Leptospira* spp., and *E.cuniculi* in the following chapters 4, 5 and 6.

Chapter 4. *Coxiella burnetii*

4.1 Introduction

The first pathogen selected to investigate the proof of principle that carnivores can act as sentinels for infection in the prey species that they consume was *Coxiella burnetii*. *C. burnetii* is an obligate intracellular bacterium that causes Q fever, which is recognised as a worldwide zoonosis with extensive animal reservoirs including mammals, birds and arthropods, particularly ticks (Angelakis and Raoult, 2010). *C. burnetii* is classified as a Group 3 pathogen and is also recognised as a potential agent of bioterrorism (Madariaga et al., 2003). Q fever was first described in 1935 in Australia, as an outbreak of a febrile illness in abattoir workers (Derrick, 1937), and the causal agent was originally termed *Rickettsia burnetii* after its isolation from guinea pigs injected with blood or urine from affected patients (Burnet and Freeman, 1937). It was subsequently reclassified in 1948 as the only member of the genus *Coxiella*, and more recently, based on 16S rRNA sequence analysis, has been reclassified from the order *Rickettsiales* to *Legionellales,* in the gamma group of Proteobacteria that includes *Legionellae* spp, *Franciscella tularensis* and *Ricketsiella* spp. (Raoult et al., 2005). Although *C. burnetii* has a cell membrane similar to that of gram-negative bacteria, it is not usually stainable with the Gram technique, and the Gimenez method is used to stain the organism in clinical specimens or cultures (Giminez, 1964).

C. burnetii is found in two different morphological forms: a small cell variant (SCV) and a large-cell variant (LCV), which correspond to different intracellular development stages (Maurin and Raoult, 1999). SCVs are compact small rods with a dense centre of condensed nucleoid filaments and are metabolically inactive spore-like forms which are released when the cells lyse. SCVs can exist extracellularly and survive for long periods in the environment, and are resistant to chemical agents including disinfectants (Raoult et al., 2005). Once in the host, the infecting SCVs change to LCVs, which are the metabolically active, intracellular forms of *C. burnetii*. Transition between SCV and LCV is accompanied by changes in the expression of surface proteins (Waag, 2007).

C. burnetii also displays antigenic, or phase, variation (Angelakis and Raoult, 2010), Phase I is characterised by a smooth full-length lipopolysaccharide (LPS) and is highly infectious; a single organism can infect a human and cause disease (Fournier et al., 1998) and less than four phase I organisms have been demonstrated to cause disease in guinea pigs (Moos and Hackstadt, 1987). Phase I is considered the wild type natural phase of the organism that can be directly isolated from naturally infected animals, man and ticks. In contrast the avirulent phase II form is characterised by rough incomplete truncated LPS and can only be isolated after serial passages in cell cultures or embryonated egg cultures. Phase II forms of *C. burnetii* cannot establish infection in an immunocompetent host (Hackstadt, 1990; Shannon and Heinzen, 2009).

The major mode of infection is via aerosol (inhalation), but infection by ingestion of contaminated dairy products is also possible (Woldehiwet, 2004). *C. burnetii* targets alveolar macrophages and other mononuclear phagocytes within vertebrate hosts, enters these host cells passively and is internalised within phagosomes, which fuse with lysosomes and form a large parasitophorous vacuole where replication occurs (Maurin and Raoult, 1999). *C burnetii* is resistant to killing and persistent in the environment for several weeks, and can also be spread by the wind, so direct contact with animals is not necessary for infection to occur (Maurin and Raoult, 1999). In humans and other animals, the route of infection is via the respiratory or digestive tracts (inhalation or ingestion), but vertical and sexual transmission can also occur in animals (Kruszewska and Tylewska-Wierzbanowska, 1997; van et al., 1993) and has been suspected in man (Mann et al., 1986). Stray cats have been shown to have a higher seroprevalence to *C burnetii* than pet cats (Komiya et al., 2003a), and, although this has not been investigated, this may reflect greater contact with infected wild rodents, including by ingestion. Arthropods, mainly ticks, can also be naturally infected with phase I *C. burnetii* from feeding on infected hosts and experimental transmission via tick bites has been demonstrated (Maurin and Raoult, 1999), but ticks are known not to be essential in the natural cycle of infection in domestic livestock (Babudieri, 1959). One study in Spain showed that ticks were negative for *C. burnetii* in an area where the infection was endemic in wildlife, indicating that they do not necessarily play an important role in transmission (Astobiza et al., 2010).

In addition to producing an acute febrile illness in man, which can be self-limiting, *C burnetii* can produce chronic persistent infections in both man and other animals. In humans chronic infection is usually associated with immunosuppression or pregnancy and endocarditis is the major feature. Symptoms may appear months or years after initial exposure to the organism. Persistently infected animals are generally asymptomatic and in these cases the term coxiellosis is deemed more appropriate than Q fever (Lang, 1988). The organism is found in the blood, lungs, spleen and liver, and is shed in urine and faeces. During pregnancy, in both humans and other animals, there is massive contamination of the placenta with *C burnetii* which can lead to abortion or low foetal birth weight (Babudieri, 1959; Carcopino et al., 2009). The mammary glands are also infected and large numbers of bacteria can be found in milk. Laboratory animals deliberately infected with *C. burnetii* as animal models of acute Q fever, including rodents, rabbits and monkeys, can develop fever and granulomas, or die (Maurin and Raoult, 1999). Infected ruminants are the main source of infection for humans, but pet cats and dogs and other mammals, including wild rodents, and birds also represent potential, but little known, sources of infection (Babudieri, 1959). In the UK, antibodies to *C. burnetii* have been detected in wild brown rats on farms (Webster et al., 1995b).

Based on the above, *C. burnetii* was selected for investigation of the proof of principle being tested in this study due to

- its wide animal host range, including both target carnivores and prey species for this study (Babudieri, 1958),
- ingestion being recognised as one means of infection (Woldehiwet, 2004),
- importance as both a zoonosis and cause of reproductive losses in domestic livestock (Woldehiwet, 2004), and
- recent increase in reported cases in humans, domestic ruminants and wildlife in many parts of the world, (Amitai et al., 2010; Enserink, 2010; Koch et al., 2010; Lemos et al., 2010; Ruiz-Fons et al., 2008; van der et al., 2010; van et al., 2010; Wallensten et al., 2010), including the UK (Wallensten et al.,

2010).

The reliance on serological testing in animals for evidence of exposure (OIE, 2008b), also made it a suitable candidate for this study.

4.2 Test options for *C. burnetii*

In humans, diagnosis of Q fever can be based on clinical symptoms (acute infection: fever, pneumonia, hepatitis, cardiac involvement, skin rash and neurologic signs; chronic infection: endocarditis), culture (within biosafety level 3 laboratories), immunodetection, and PCR-based assays, but in most instances still relies on serology (Angelakis and Raoult, 2010). A variety of serological techniques exist, but in humans the indirect immunofluorescent antibody (IFA) test (see 4.2.1) is most commonly used (Fournier et al., 1998). Other serological techniques include the complement fixation test (CFT) (see 4.2.2), enzyme-linked immunosorbent assay (ELISA) (see 4.2.3), and microagglutination tests (Fournier et al., 1998).

The antigenic differences between phase I and II LPS are useful for the serological differentiation between acute and chronic Q fever. In acute infections in humans, IgM antibodies to phase II antigens appear rapidly, reach high titres within 14 days and persist for 10-12 weeks (Maurin and Raoult, 1999). IgG antibodies to phase II antigens reach a peak at approximately 8 weeks after the onset of symptoms. IgG antibodies to phase I antigens, primarily LPS, develop more slowly and remain at lower titres than those to phase II antigens. Seroconversion or a four-fold rise in titres indicates acute infection. In chronic Q fever in humans, where the organism persists, IgG titres to phase I and phase II may be high and there may also be presence of IgA antibodies to phase I. Serology is used to follow treated patients to determine if treatment is successful and to enable early diagnosis of chronic infections (Landais et al., 2007). Thus high anti-phase II antibody titres are considered diagnostic for acute infection, and high anti-phase I antibodies diagnostic for chronic infection (Fournier et al., 1998).

However, infection in animals is usually asymptomatic and diagnosis of infection can be based on direct identification of the organism or serological tests, depending on the type of sample and the purpose of the diagnosis (OIE, 2008b). In contrast to

humans, phase I and phase II antibody responses and immunoglobulin class responses have not been well studied in other animals (OIE, 2008b), but the presence of IgG antibodies against phase II antigens is most commonly used to provide evidence of a recent infection with *C. burnetii* or past exposure (OIE, 2008b).

Direct identification of the organism is useful in determining the cause of abortions in domestic ruminants, and, if suspected as being caused by *C. burnetii*, direct staining (Stamp, Giminez or Machiavello methods) and microscopic examination of placental cotyledons or tissues from the aborted foetus may be carried out. If *C. burnetii* is detected microscopically, then serological testing is used to confirm the diagnosis (OIE, 2008b). Specific detection can also be achieved using immunodetection or DNA amplification. Immunohistology on paraffin-embedded tissues or acetone-fixed smears can be achieved with indirect immunofluorescence or immunoperoxidase assay using polyclonal *C. burnetii* antibodies, a human antiserum or one produced in laboratory animals, but no specific antibodies for these techniques are available commercially (OIE, 2008b). PCR methods are still under development but have been used to detect *C. burnetii* DNA in cell cultures and biological samples such as genital swabs, milk and faeces (Berri et al., 2000). Demonstration of *C. burnetii* by immunohistology or PCR has been proven to be more specific and sensitive than standard histological techniques (OIE, 2008b).

Isolation of *C. burnetii* can be performed by inoculation of suspected infected material into embryonated chicken eggs or cell culture followed by staining, or in some cases into laboratory rodents followed by serological testing and microscopic or PCR examination of organ samples (OIE, 2008b). Isolation using laboratory animals can be used where isolation of *C. burnetii* from tissues contaminated with various microorganisms is required, or in order to obtain phase I antigens (OIE, 2008b).

For the current study, serological testing was selected, and this has already been widely used for seroprevalence studies in both wildlife species and domestic cats (Burgdorfer et al., 1963; Ejercito et al., 1993; Enright et al., 1971; Komiya et al., 2003a; Marrie et al., 1986; Marrie et al., 1993; Matthewman et al., 1997; Ruiz-Fons et al., 2008; Shannon and Heinzen, 2009; To et al., 1998; Webster et al., 1995b;

Webster and Macdonald, 1995; Zarnke, 1983). Although both serological responses and direct bacterial evidence are necessary for establishing definitively the presence of infection in individual animals, serological testing alone is useful for screening groups or herds and determining prevalence (OIE, 2008b). The three serological tests most commonly used in animals are the IFA, the ELISA and the CFT. Other older tests no longer used in routine diagnosis are the microagglutination tests, the capillary agglutination tests and the indirect haemolysis test (OIE, 2008b).

The microagglutination test (Fiset et al., 1969) is simple and more sensitive than the CFT (Kazar et al., 1981) but requires large amounts of antigen. Test serum is incubated with a solution of purified phase II *C. burnetii* antigen and presence or absence of agglutination is visually assessed by the sedimentation pattern, often assisted by addition of a dye to the antigen. Non-agglutinated serum forms a discrete sharp button at the base of a microplate well, whereas agglutinated serum either does not sediment or forms a diffuse poorly defined sediment. The microagglutination test has been used to detect antibodies to *C. burnetii* in domestic and wild birds, including crows, in Japan (To et al., 1998).

The majority of serologic tests designed for human or livestock pathogens, such as the one used in this study, have not been validated for use in non-target species (Greiner and Gardner 2000) and have been directly transposed from use in domestic livestock species (Gardner et al., 1996).

4.2.1 Indirect immunofluorescence assay (IFA)

The IFA is the reference method for Q fever serological testing in humans (Maurin and Raoult, 1999) and has been adapted for use in animals (Woldehiwet, 2004). The basis of the test is that plates or slides with fixed antigen are incubated with test serum, then secondary antibodies labelled with a fluorochrome are added and the antigen-antibody reaction visualised by fluorescent microscopy (Burr and Snodgrass, 2004). Both phase I and II antigens are used from the reference strain (Nine Mile strain; ATCC VR 615, obtained from the first American isolate of *C. burnetii* from a tick); phase I is obtained from spleens of infected mice and phase II by growing *C. burnetii* in cell culture. Commercial kits with antigen-spot slide wells containing either phase I only or both phase I and II antigens (*e.g.* Coxiella burnetii-SpotIF™,

bioMerieux; Q fever IFA Test Kits™, MRL Diagnostics) can be adapted by replacing the human spp. conjugate by a species specific conjugate or protein A/G.

The IFA has been used to investigate seroprevalence of *C. burnetii* in domestic cats (Komiya et al., 2003a; Matthewman et al., 1997), brown rats (Webster et al., 1995b),deer (Ruiz-Fons et al., 2008), hares, moose, and raccoons (Marrie et al., 1993). In man, the IFA has a reported low sensitivity of 58.4% but a specificity of 92.2% for acute infections, and a 100% sensitivity for chronic infections (specificity not assessed) (Dupont et al., 1994). It has also been shown to have higher specificity and sensitivity than the ELISA (Slaba et al., 2005). This high sensitivity for chronic infections makes the IFA very suitable for seroprevalence studies such as the current study, where the aim is to detect chronically infected asymptomatic animals; however the disadvantage of the IFA is that it is labour intensive and cannot be automated, requiring the use of specialist microscopes and experienced staff (Burr and Snodgrass, 2004).

4.2.2 Complement fixation test (CFT)

The CFT uses the ability of antibody bound to antigen to fix complement and inactivate it. If complement is not fixed, the addition of sensitised red blood cells (erythrocytes) will result in their lysis, thus absence of erythrocyte lysis indicates a positive result. Sheep erythrocytes, sensitised by coating them in anti-sheep antibody, are most commonly used. The CFT thus does not require the use of species-specific antibodies. It is very specific (Fournier et al., 1998), although less so than the IFA or ELISA, but it lacks sensitivity (73 - 77.8% (Field et al., 2000; Peter et al., 1987). Nevertheless, the CFT can give excellent results for routine diagnosis at the flock level for abortive diseases and is still widely used in many countries. It is the OIE prescribed serological test and in the UK it is offered commercially for domestic ruminants by the Government's Veterinary Laboratories Agency.

In wildlife, the CFT has been used to determine seroprevalence of *C. burnetii* in a variety of species including bears, coyotes, caribou, moose, feral cats, bobcats, foxes, mustelids, wild rodents and marsupials (Binninger et al., 1980; Enright et al., 1971; Munday, 1972; Zarnke, 1983). However the CFT is even more time

consuming and labour intensive than the IFA (18 hours vs. 1 hr incubation) and the antigen used often fails to detect antibodies in sheep or goats (Angelakis and Raoult, 2010; Burr and Snodgrass, 2004; Fournier et al., 1998; OIE, 2008b).

4.2.3 Enzyme-linked immunosorbent assay (ELISA)

The ELISA is similar in principle to the IFA but has the advantage of being easily automated and thus lends itself to dealing with large numbers of samples. Test serum is incubated with antigen that is immobilised in wells on a test plate. Secondary antibodies labelled with an enzyme marker are then added, and after washing, bound secondary antibodies can be detected by a colour change induced by addition of the enzyme substrate (Burr and Snodgrass, 2004). In humans the ELISA was originally proposed as a good method for seroepidemiological surveys (Peter et al., 1987) but has also been shown to be useful for the serodiagnosis of Q fever (Frangoulidis et al., 2006; Peter et al., 1988). For diagnosis in animals, commercial ELISA kits for ruminants are widely available and microplate wells are coated with whole-cell inactivated antigen. Currently, kits can detect either anti-phase II antibodies, or both anti-phase I and anti-phase II antibodies (OIE, 2008b).

The ELISA has been used in wildlife species, including black bears, various deer species, hares, monkeys, wild rats, raccoon dogs, serow, wild pigs and palm civets in Japan, where species-specific conjugated antibodies are not available and have been replaced with conjugated Protein A and G (Ejercito et al., 1993). Reports of its use cannot be found in species of rodents other than rats or nutria (*Myocastor coypus*), nor in foxes or domestic cats. The ELISA has a high sensitivity and good specificity. For example in the diagnosis of acute Q fever in man, the ELISA has a reported sensitivity of 80% for anti-phase II IgG and 84% for anti-phase II IgM and a specificity of >99% in one study (Waag et al., 1995), and in a other study a specificity of 97.7% and sensitivity of 95.65% for anti-phase II IgM (Frangoulidis et al., 2006).

4.3 Test selection and modification

An ELISA test for *C. burnetii* was selected for this study, as this test:

- is available commercially in kit form,
- can be automated to enable the processing of large numbers of samples,
- does not require the use of a specialised UV microscope or specific expertise that underpins the IFA,
- is much less labour intensive and more specific and sensitive than the CFT.

Because the commercial ELISA kit selected was designed for testing ruminant species, and thus uses anti-ruminant peroxidase as the conjugate, the manufacturers of the selected test kit (IDVet, Montpellier, France) were contacted to discuss modification of their kit to be able to test multiple wild species by replacing the anti-ruminant peroxidase with a protein A and G conjugate, as in the Japanese study by Ejercito *et al.* (1993). Modified prototype kits were supplied by IDVet specifically for this study.

The main purpose of using the ELISA test was to use the optical density (OD) and sample:positive control percentage (S/P) results to generate information on seroprevalence of antibodies to *C. burnetii* in the species investigated. Interpretation of S/P values is dependent on the threshold at which a sample is deemed positive. The thresholds for domestic ruminants used in the commercial test employed in this study were determined by the manufacturers by analysing the optical densities of serum from known positive and negative bovine populations, then placing the cut-off at an S/P between these two populations where overlap is minimised (A. Lecoq, IDVet Ltd., pers. comm.). This is a common approach for determining thresholds for ELISA tests(Gardner et al., 1996; Greiner et al., 1994).An alternative approach is to use the mean value from a known negative reference population and add a two-or three-fold standard deviation to the mean to this value to determine the threshold (Richardson et al., 1983).

Until a threshold level is decided upon, seroprevalence information cannot be derived from the serological test results, and thus seroprevalence in predator and prey species cannot be compared. The test used in this study had a threshold of 50% (as set by the manufacturer for domestic ruminants); however the test has not been

used or validated for wild rodent or carnivore species, or for domestic cats

4.3.1 Materials and methods

The modified commercial indirect ELISA kit, adapted for use in non-ruminant species, was used (IDVet, Montpellier, France). The kit consisted of 96-microwell plates pre-coated with phase I and phase II antigens, from a *C. burnetii* strain isolated in France from an aborted bovine placenta. The kit was adapted to allow detection of a wide spectrum of mammalian immunoglobulin G (IgG) using a mixture of protein A and G to detect bound serum (LeCoq, personal communication), in order to maximise its utility for testing rodent, fox and cat sera. However, this did not enable its use for testing avian serum, as avian IgY does not bind to protein A (Kronvall et al., 1974) or protein G (Akerstrom et al., 1985), and so corvid sera was not tested.

The testing procedure was conducted as follows for each 96-microwell plate:

1. All reagents were allowed to come to room temperature before use, and homogenised using a bench-top Vortex.
2. 90 µl of Dilution Buffer 2 was added to each microwell.
3. 10 µl Negative Control was added to wells A1 and B1, 10 µl Positive Control was added to wells C1 and D1, and 10 µl of each test serum was added in duplicate to the remaining wells (46 samples per plate).
4. The plate was incubated for 45 minutes at room temperature
5. Wells were washed with approximately 300 µl Wash Solution three times
6. 100 µl Conjugate was added to each well, and the plate incubated for 30 minutes at room temperature
7. Wells were washed with approximately 300 µl Wash Solution three times
8. 100 µl Substrate Solution was added to each well and the plate incubated for

15 minutes at room temperature in the dark

9. 100 µl Stop Solution was added to each well and the optical densities measured on a Model 550 microplate reader (Bio-Rad Laboratories, Hemel Hempstead, UK) reader at 450nm

In accordance with the manufacturer's instructions, the test was validated if the mean optical density (OD) of the Positive Control (OD_{PC}) was > 0.350 and the ratio of the mean OD values of the Positive and Negative controls was > 3. For each sample the sample:positive control (S/P) percentage was calculated using the formula provided by the manufacturer where the raw OD values were normalised by adjusting each sample to the mean values obtained for the positive and negative controls obtained on the specific plate as follows:

$$S/P = \frac{OD_{sample} - OD_{nc}}{OD_{pc} - OD_{nc}} \times 100$$

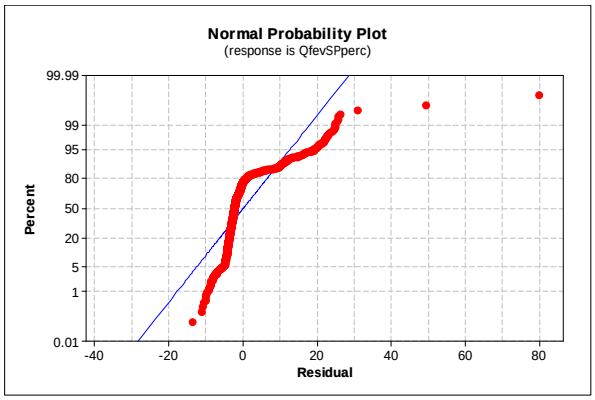
According to the manufacturer, when used for domestic ruminants an S/P of < 40% is considered negative, between 40 and 50% is doubtful, between 50 and 80% positive and >80% strong positive. In the current study, cut-off, or threshold values, were explored in order to then convert S/P values into ELISA-positive or ELISA-negative results to investigate seroprevalence. A more conventional approach of evaluating S/P data was then adopted by determining if the distributions fall into two distinct populations representing those with background reactivity to the ELISA test (negative) and those with specific antibodies to *C. burnetii* (positive).

Data analysis

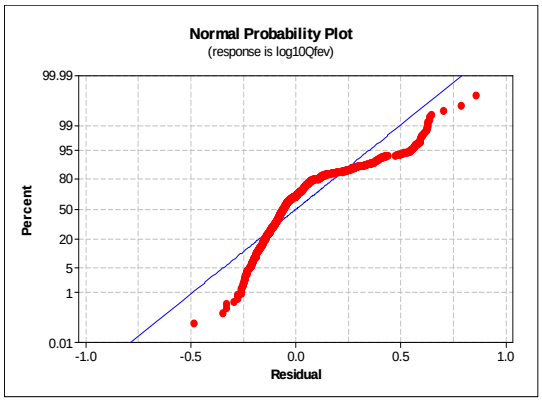
Statistical analysis of the results was performed using Minitab 15® and R® (R Foundation). The intention was to use general linear mixed effect modelling on the raw S/P values, however normality of the residuals was not achieved (Figure 4.1).

Figure 4.1 Probability plots for residuals for a) raw S/P and b) log10 S/P values for the *C. burnetii* ELISA test.

a)



b)



Kruskal-Wallis analysis was therefore used to test for differences in S/P values between predator and prey species overall, by individual species and by study area. Post hoc analysis of all pairwise comparisons (Dwass-Steel-Critchlow-Fligner method) (Hollander and Wolfe D.A, 1999) was used for differences in S/P values between predator and prey species and was performed using StatsDirect® Version 2.7.8 (StatsDirect Ltd). Summary variables of the S/P results (mean, median, minimum, maximum, Q1, Q3) were explored for relationships between prey and predator species, and Pearson's correlation coefficient (*r)* used to see if any associations were statistically significant.

For seroprevalence data, generalised linear mixed effect modelling with binomial errors was used to explore seroprevalence in prey and predator species, with study area as a random effect for all species, and study site nested within study area for prey species. Fixed effects of sex, age, and season were incorporated. For age, animals were classed as either adult or non-adult (juvenile and subadult). For all tests used, the significance level was placed at $P<0.05$.

4.4 Results

A total of 924 serum samples were tested for antibodies to *C. burnetii*, from 796 prey species (180 bank voles, 309 field voles, 307 wood mice) and 131 predator species (26 cats, 105 foxes) . This represented 87.3% of the total number of samples of these

five species that were collected during the study (Table 4.1). The majority of samples for both prey and predators were collected in seasons 1 and 2, and in season 4 only the Cumbria study area was sampled for prey species.

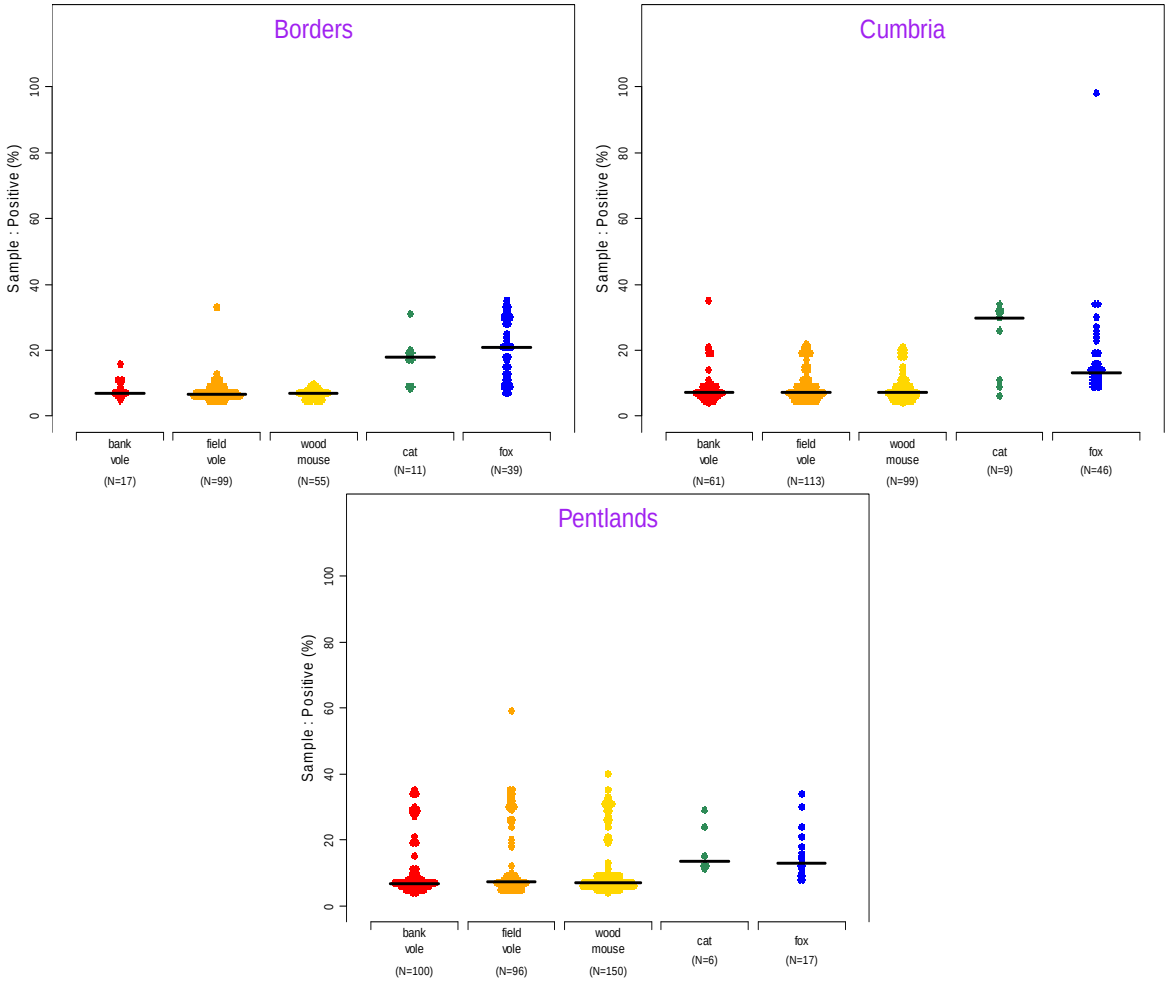
Table 4.1 Numbers of serum samples tested for *C. burnetii* by area, predator or prey status, species and season

Area	Predator Species	Total tested	Season 1	Season 2	Season 3	Season 4	Non seasonal
Borders	Cat	11	0	0	0	0	11
	Fox	39	24	4	6	5	
Cumbria	Cat	9	0	0	0	0	9
	Fox	46	1	24	9	12	
Pentlands	Cat	6	0	0	0	0	6
	Fox	17	9	2	2	4	
		128	**34**	**30**	**17**	**21**	**26**
	Prey Species						
Borders	Bank vole	17	5	12	0	0	
	Field vole	99	47	45	7	0	
	Wood mouse	55	22	3	30	0	
Cumbria	Bank vole	61	11	25	20	5	
	Field vole	113	27	45	19	22	
	Wood mouse	99	39	47	9	4	
Pentlands	Bank vole	102	44	46	12	0	
	Field vole	97	19	52	26	0	
	Wood mouse	153	78	38	37	0	
		796	**292**	**313**	**160**	**31**	
	TOTAL	**924**	**326**	**343**	**177**	**52**	**26**

4.4.1 Raw S/P values

Initially the raw data, expressed as sample:positive control (S/P) %, was explored for each species, without consideration as to whether or not the S/P value represented a positive or negative sample . Predator median S/P values were significantly higher ($P<0.001$) than prey median S/P values overall, and by study area (Figure 4.2).

Figure 4.2 Plots of S/P values for each species per site. Horizontal bar represents the median value. These dot plots have been jigged so that width of the "blob" of overlying dots reflects, but is not an exact representation of, the numbers of animals with this S/P value.



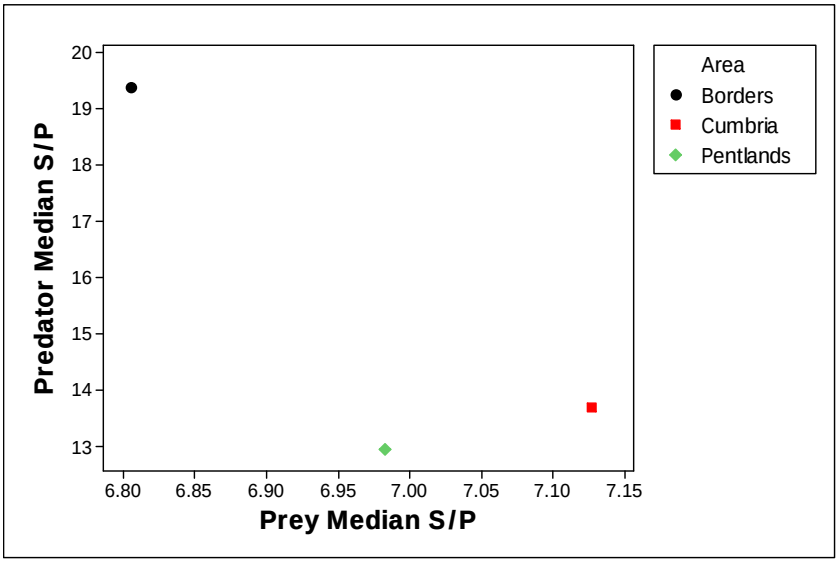
At an individual species level for the study overall, median S/P values were remarkably similar at 6.960-6.965 for all three prey species (Table 4.2) There were no significant differences between cats and foxes (P=0.956) or between any of the three rodent prey species (P>0.972), but there were significant differences between predator species (cat and fox) and all prey species (P<0.001). This pattern of a significantly higher seroprevalence in predator than in prey species (P<0.001) was also consistent in each study area.

Table 4.2 Median S/P values (with ranges) and mean (with SE mean) values for *C. burnetii* ELISA test results in prey and predator species.

Species	Median S/P	Mean S/P
	Overall	
Bank vole	6.965 (4.409 - 35.124)	9.451 (0.508)
Field vole	6.960 (4.583 - 58.883)	9.706 (0.424)
Wood Mouse	6.965 (4.452 - 40.083)	9.258 (0.380)
Cat	18.03 (5.57 - 33.68)	19.27 (1.76)
Fox	13.93 (7.12 - 98.10)	18.20 (1.12)
	Borders	
Bank vole	7.07 (5.391 - 15.829)	8.059 (0.630)
Field vole	6.641 (4.627 - 33.058)	7.244 (0.302)
Wood Mouse	6.938 (4.583 - 10.455)	6.808 (0.173)
Cat	17.91 (7.91 - 30.99)	16.98 (1.97)
Fox	20.77 (7.12 - 35.12)	20.65 (1.43)
	Cumbria	
Bank vole	7.124 (4.409 - 34.915)	8.509 (0.6410)
Field vole	7.230 (4.583 - 22.359)	8.798 (0.401)
Wood Mouse	7.112 (4.452 - 20.576)	8.452 (0.405)
Cat	29.82 (5.57 - 33.68)	23.50 (3.84)
Fox	13.17 (9.23 - 98.10)	17.10 (2.04)
	Pentlands	
Bank vole	6.88 (4.452 - 35.124)	10.246 (0.798)
Field vole	7.26 (4.67 - 58.88)	13.28 (1.15)
Wood Mouse	6.960 (4.496 - 40.083)	10.661 (0.694)
Cat	13.51 (11.49 - 29.34)	17.12 (3.10)
Fox	12.95 (8.03 - 34.30)	15.55 (1.86)

Overall, the raw S/P data is in agreement with the bioconcentration concept and median S/P values are higher in predators than in prey. One potential outcome of this concept is that there would be a positive relationship between S/P values in prey and those in predators. Thus in areas with higher values in prey there would also be correspondingly higher values in predators, provided that other factors in each area that might affect exposure to a pathogen, such as population size, demographics, habitat etc. were similar. However, exploration of the raw data for possible relationships between summary variables for prey and predator species in each study area (mean, median, minimum, maximum, Q1, Q3) did not reveal any obvious positive patterns, for example Figure 4.3.

Figure 4.3 Relationship between median S/P values in predators and prey



Season and lagged season

Relationships between S/P values in predator and prey were also explored in terms of season, but no consistent positive patterns were found. Cats were not examined in this way because they were not allocated a sampling season. Because of the timeline of rodent sampling in relation to fox sampling (See Chapter 3, Figure 3.18), the possibility of a lag effect between prey and fox seroprevalence was also explored, *i.e.* a delay in the possible influence of prey S/P values on those in predators. This was only done for the Cumbria area, which had 4 seasons; in Borders and Pentlands areas only three seasons were used, and thus only two points could be plotted for the lagged season so effects of lagged season could not be assessed. Relationships between summary variables of S/P data were explored by comparing values from one season in prey with the following season in predators (*e.g.* seasons 1,2,3 in Cumbrian prey species were compared with seasons 2,3,4 in Cumbrian foxes respectively), but again no consistent or significant relationships were found (data not shown).

Summary of raw data results

On examination of the summary variables for the raw S/P data it was found that median values were significantly higher in predators than prey in all areas

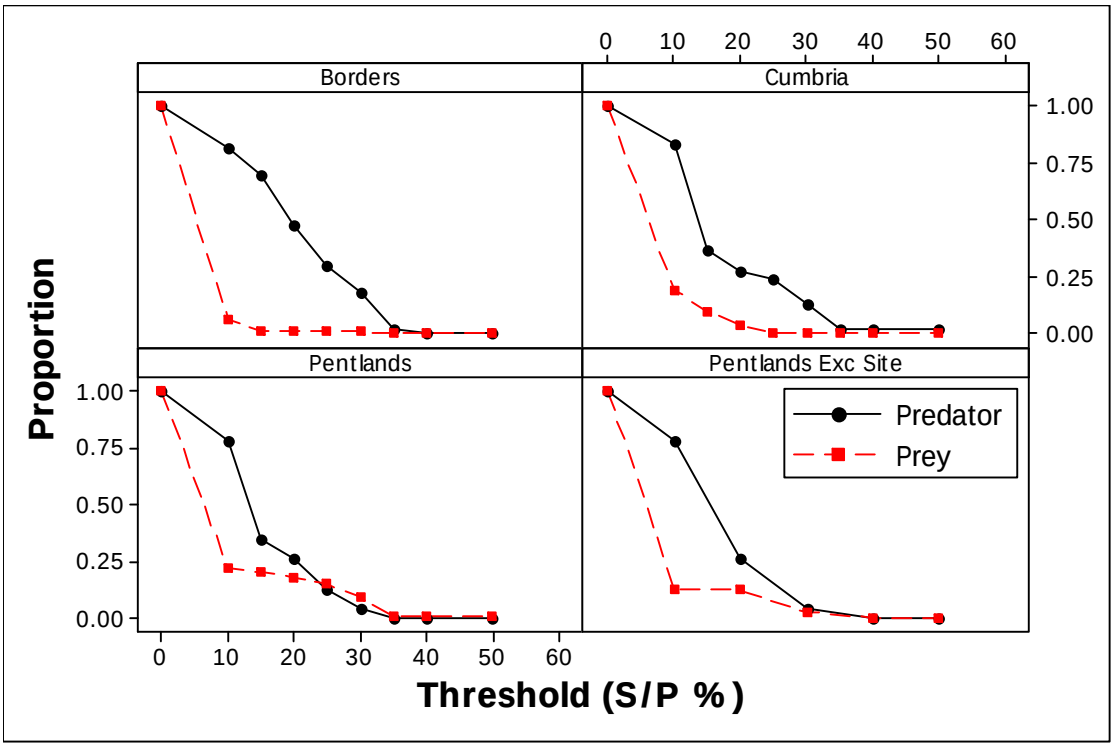
consistently and irrespective of species. Median S/P values did not differ significantly between individual predator species (cats and foxes) or individual prey species (voles and wood mice). In contrast, no consistent patterns were found relating prey and predator S/P values by area, season or lagged season.

4.4.2 Thresholds

In the absence of known positive and negative populations for each of the five species tested, thresholds were firstly explored in a simple fashion by using the test threshold of 50% and lower thresholds (10%, 20%, 30%, 40%) to look at proportions of prey and predator species at each, and to explore possible relationships between predators and prey at differing threshold levels.

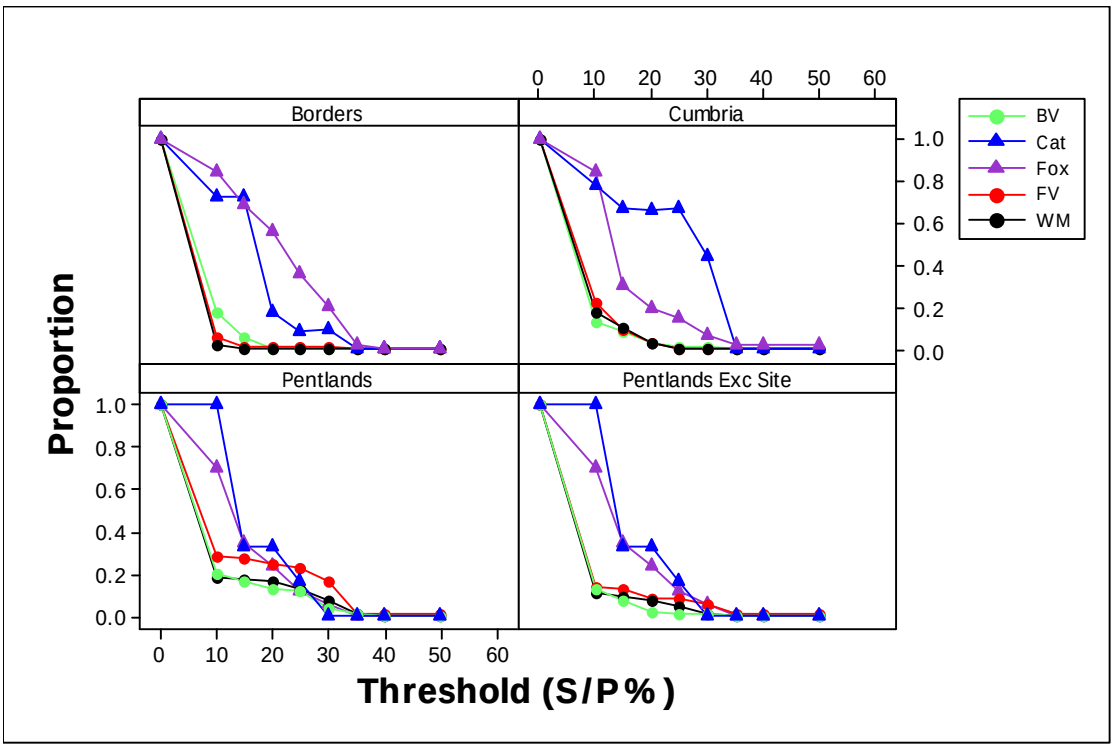
Overall, at each threshold the predator species have a higher proportion of "positive" animals, (*i.e.* those above that particular threshold) (Figure 4.4), up to the 35% threshold in Borders and Cumbria and up to the 25% threshold in Pentlands, above which the proportion of predators are similar to that of prey.

Figure 4.4 Proportions of predator and prey species above differing S/P thresholds in each study area. (Pentlands Exc Site refers to the Pentlands site with one study site accounting for 63% of all S/P values > 15% excluded)



Looking at the individual prey and predator species level (Figure 4.5), in the Borders and Cumbria sites there is a clear pattern of a higher proportion of predator species (cat, fox) than prey species at each threshold level up to 30%. In the Pentlands site this pattern is also present up to 20% with the exception of field voles, the proportion of which is similar to that of foxes at the 20% threshold, and greater than that of foxes from 20% to 35% thresholds. Further exploration of the Pentlands data revealed that one particular study site out of the 6 used accounted for 63% of all S/P values > 15% in prey species (all in seasons 2 and 3) meaning that proportions of prey were above those of predators above this threshold in this study area. If this site was excluded (Pentlands Exc Site Figure 4.5) from analysis a similar pattern to the Borders and Cumbria study areas was observed, indicating that an outbreak or epidemic could have occurred in this particular site over Autumn/Winter 2007/8 and Summer 2008.

Figure 4.5 Proportion of each individual species above differing thresholds in each study area (BV = bank vole, FV = field vole, WM = wood mouse). (Pentlands Exc Site refers to the Pentlands site with one study site accounting for 63% of all S/P values > 15% excluded)



The effect of season on the proportion of each species at or above each threshold was also explored, to see if the proportion of animals above a particular threshold might be influenced by the proportion in the preceding season. However, no consistent patterns were apparent (data not shown).

"Cut-off", or threshold, determination

Examination of the distribution of S/P values for each of the species indicates that, for the three rodent species (Figure 4.6 a), two populations are present. For all three species overall and in all areas there is a peak response between 0 and 10% S/P, which is likely to correspond with the negative population. However, between approximately 15% and 100% there appears to be a second smaller population, which could represent the positive population. This second population was present for all species, except for field voles in the Borders area (Fig 4.6a).Thus for this study it was determined that the threshold should be placed at 15% in these rodent species.

For the predator species (Figure 4.6 b) the numbers tested are much lower, especially cats (cat N= 26, fox N= 102) and the population distributions are not as distinct as for the rodents. However, there are still two patterns of distribution overall and in all three areas, and a threshold could be placed at 25%. For foxes in Cumbria, this threshold is least distinct and the distribution suggests it may be slightly lower (20%), however using the higher threshold should still distinguish suspected positive samples.

On the basis of these distributions, a test threshold of 15% for prey and 25% for predators was used in this study to determine seroprevalence.

Figure 4.6 a) Distribution of S/P values for prey species. Dotted line at 15% indicates the selected threshold that appears to separate two populations

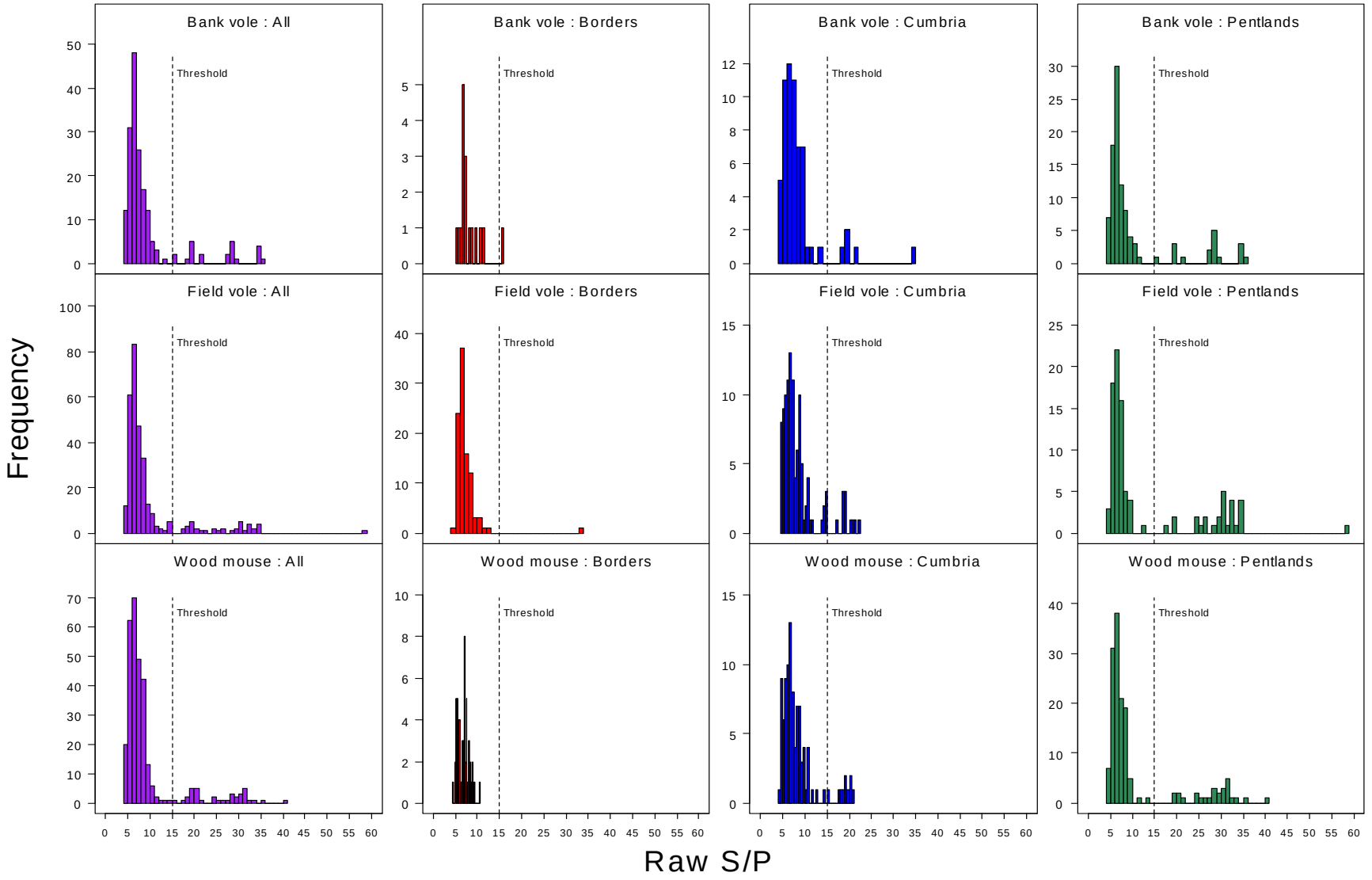
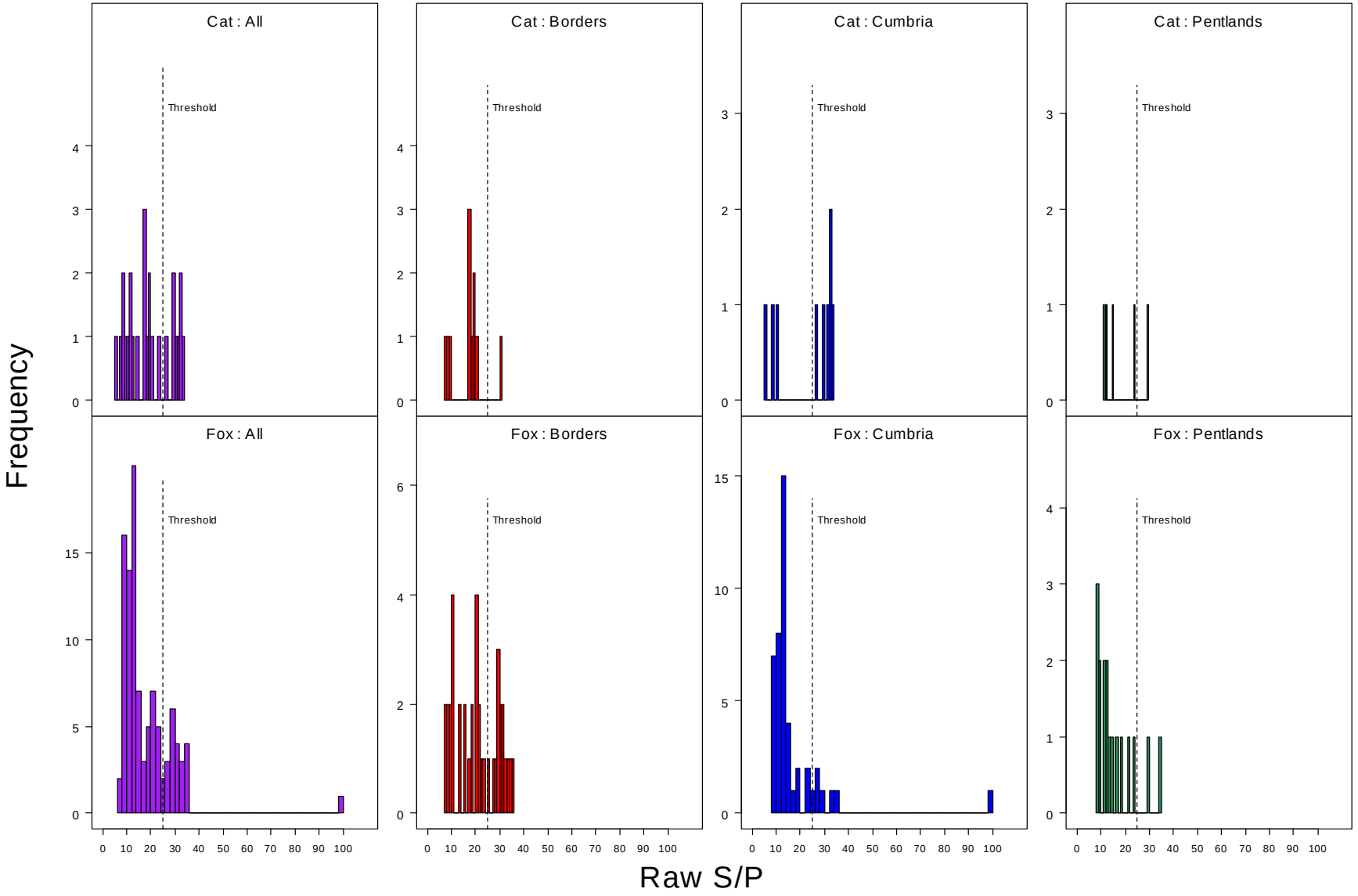


Figure 4.6b) Distribution of S/P values for predator species. Dotted line at 25% indicates the selected threshold that appears to separate two populations



4.4.3 Seroprevalence

Using the test threshold, as with the raw S/P values, seroprevalence was significantly higher in predators (24.22%) than in prey (12.44%) for the study overall ($p<0.001$) and between individual prey and predator species, ($P<0.035$), irrespective of study area (prey 0 – 27.84%; predators 9.09 – 66.67%) (Tables 4.3 and 4.4).There were no significant differences in seroprevalence between individual prey species ($P >0.832$), nor between cats and foxes ($P=0.916$).

Table 4.3 Seroprevalence for *C. burnetii* (with 95% confidence intervals) for predators and prey overall

Predator/ Prey	Species	N	Seroprevalence
Prey	Bank vole	180	12.78 (8.28 - 18.55)
	Field vole	309	12.62 (9.13 - 16.85)
	Wood mouse	307	12.05 (8.63 - 16.23)
	All	**796**	**12.44 (10.22 - 14.93)**
Predator	Cat	26	30.77 (14.33 - 51.79)
	Fox	102	22.55 (14.86 - 31.89)
	All	**128**	**24.22 (17.09 - 32.58**

Table 4.4 Seroprevalence for *C. burnetii* (with 95% confidence intervals) for prey and predator species in each study area

Predator/Prey	Species	Area	N	Seroprevalence
Prey	Bank vole	Borders	17	5.88 (0.15 - 28.69)
		Cumbria	61	8.20 (2.72 - 18.10)
		Pentlands	102	16.67 (10.02 - 25.34)
Prey	Field vole	Borders	99	1.01 (0.02 - 5.50)
		Cumbria	113	9.73 (4.96 - 16.75)
		Pentlands	97	27.84 (19.21 - 37.86)
Prey	Wood mouse	Borders	55	0.00 (0.00 - 5.30)
		Cumbria	99	10.10 (4.95 - 17.79)
		Pentlands	153	17.65 (11.96 - 24.63)
Prey	Overall	Borders	171	**1.17 (0.34 - 9.80)**
		Cumbria	273	**9.52 (6.32 - 13.64)**
		Pentlands	352	**20.17 (16.10 - 24.75)**
Predator	Cat	Borders	11	9.09 (2.30 - 41.28)
		Cumbria	9	66.67 (29.93 - 92.51)
		Pentlands	6	16.67 (0.42 - 64.12)
	Fox	Borders	39	35.89 (21.20 - 52.82)
		Cumbria	46	15.22 (6.34 - 28.87)
		Pentlands	17	11.76 (1.46 - 36.44)
Predator	Overall	Borders	50	**30.00 (17.86 - 44.60)**
		Cumbria	55	**23.64 (13.23 - 37.02)**
		Pentlands	23	**13.04 (2.78 - 33.59)**

Seroprevalence in both prey and predators varied between study areas, from 1.17 – 20.17% in prey and 13.04 – 30.0% in predators (Table 4.4) and in one area, Pentlands, was higher in prey than predators.

For prey species overall, seroprevalence differed significantly between males (28/404; 6.9%) and females (P<0.001) and was higher in females (71/382; 18.6%). In individual prey species field vole seroprevalence was significantly higher in females, compared to bank voles (P=0.046), but not to wood mice (P=0.175). For predators, seroprevalence was not significantly different between males (16/62; 25.8%) and females (14/41; 34.1%) (P>0.37).

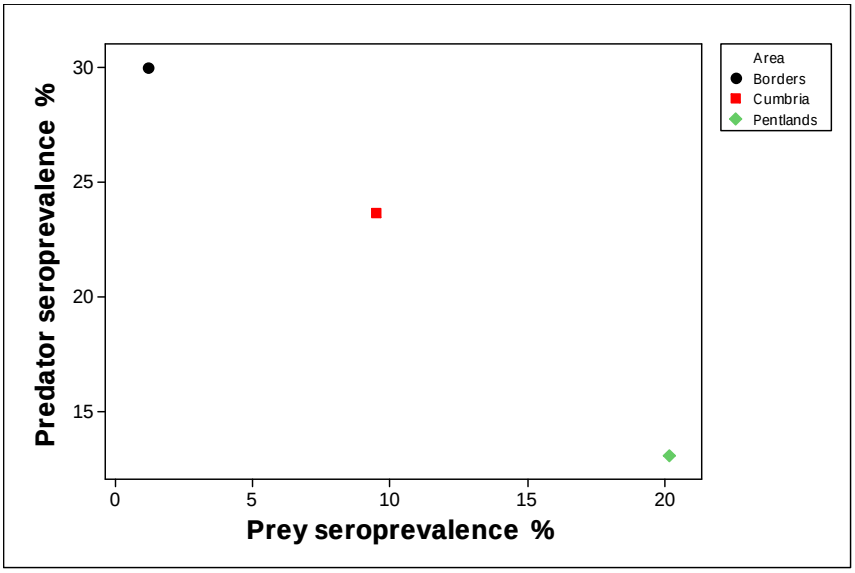
Age was not associated with a significant difference in seroprevalence in prey species (adults 98/766; 12.8%, non-adults 1/21; 4.8%) (P=0.765), but only one non-adult prey animal (field vole) was seropositive. In predators, seroprevalence in foxes was not significantly different between adults (12/44; 21/4%) and non-adults (9/15; 37.5%), however all the cats tested were adults.

In prey there were significant differences in seroprevalence depending on season for the study overall (P<0.001), (being higher in seasons 2 and 3 compared to season 1, but not between seasons 2 and 3). Season 4 was excluded from analysis as it only applied to the Cumbria study area. In prey, seroprevalence rose sequentially over seasons 1-3 from 2.45% (0.99 – 4.98) to 14.68% (10.96 – 19.211) to 20.22% (15.19 – 28.41) (p<0.001 between season 1 and 2 and seasons 1 and 3). In predators there was no significant seasonal difference in seroprevalences (P>0.54).

Patterns of seroprevalence

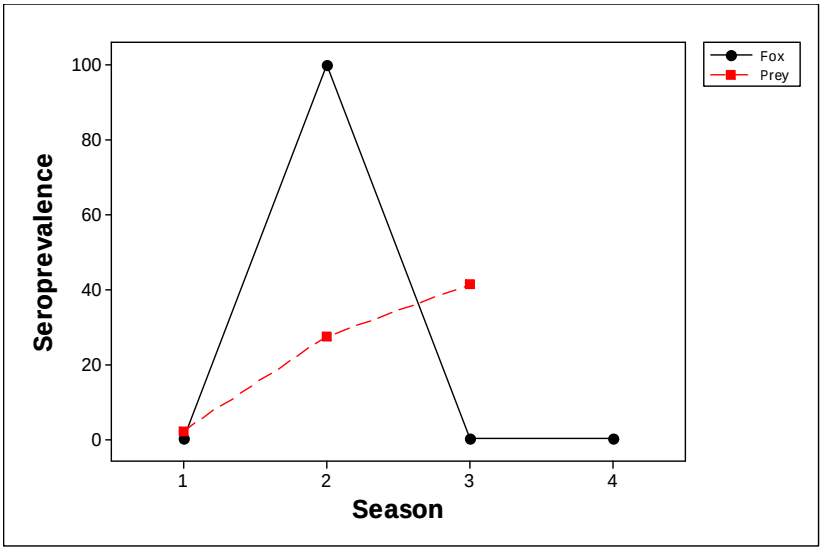
As found with the raw S/P values, plots looking at the pattern of seroprevalence between the three study areas did not show any positive patterns. In fact, a statistically significant negative relationship between predators and prey was apparent (r = -0.99, P=0.047) (Figure 4.7).

Figure 4.7 Relationship between seroprevalence in predators and prey in the three study areas



Similarly, no consistent positive patterns were found relating seroprevalence in prey to that in foxes with respect to season (cats were not assigned a season), either for the study overall, or by area (data not shown). In the Pentlands, seroprevalence in prey rose continuously over seasons 1-3, but while seroprevalence in foxes rose between seasons 1 and 2 from 0% to 100%, coinciding with a rise in prey seroprevalence in prey from 2.1% to 27.1%, they then dropped again in seasons 3 and 4 to 0% (Figure 4.8).

Figure 4.8 Seroprevalence by season in foxes and prey in the Pentlands area



Summary of seroprevalence results

In summary, it was found that seroprevalence for *C.burnetii* was significantly higher in predators than in prey, and this pattern was consistent regardless of individual species or study area. Age and sex were not significant factors affecting seroprevalence, with the exception of field voles, where females had higher seroprevalence than males. Patterns of seroprevalence comparing prey and predators varied by area and season and no consistent positive patterns relating seroprevalence in predators and prey were found.

4.5 Discussion

The purpose of this part of the study was to use an ELISA test on the serum of prey and predator species to investigate exposure to *C. burnetii*, in order to determine if exposure in predators is higher than in their prey, thereby indicating bioconcentration by ingestion or close contact. Once the ELISA test threshold was determined, by examining the distribution of the raw data and setting the threshold at where there was a distinction between two populations, the study found there was a significantly higher seroprevalence of *C. burnetii* in predators than in prey for the study overall and in each study area. This finding was also backed up by the raw S/P data, where differences in the median S/P values were significantly higher in predators than in prey overall for the study overall and in each study area. These findings are consistent with the bioconcentration concept and the hypothesis for this study.

The pattern of a negative relationship between predator and prey seroprevalence in the three areas was unexpected, and a higher overall prey seroprevalence was not associated with a correspondingly higher seroprevalence in predators. This could be due to many possible factors, including the intrinsic differences in study areas selected in terms of habitat type, prey and predator population density, distribution and home range size, and presence and degree of direct or indirect contact with other possible sources of *C. burnetii* infection such as domestic livestock. In studies of other pathogens, such as hantavirus in wild rodents population density and seroprevalence have been shown to be dependent on habitat type (Olsson et al., 2005) (Heyman et al., 2009). Seroprevalence in prey was highest (20.17%) in the Pentlands area, and in this one area seroprevalence in predators (13.0%) was not

significantly higher. This area was the most agricultural of the study areas, and the one site within it that contained the majority (62.9%) of all the Pentlands rodents with S/P values >15% was a narrow strip of woodland in between two fields in which sheep were grazed and lambed. Exclusion of this one study site from the analysis reduced the seroprevalence in prey in the Pentlands area to 11.3 %,(7.52 – 16.12%) compared to 13.0% in predators ($\chi^2_1 = 0.062$, P=0.8). Exclusion of this site reduced prey seroprevalence but did not affect patterns of difference in prey seroprevalence by season in this area, and seroprevalence was still significantly higher in seasons 2 and 3 compared to season 1, but with no significant difference between seasons 2 and 3. Although clinical disease due to *C. burnetii* has not been reported in sheep or cattle on this farm, and no routine testing for exposure has been carried out (Alex Moir, SAC, personal communication), it is possible that these rodents could have been exposed to higher levels of *C. burnetii* in this site from close contact with ruminants or a contaminated environment (Reusken et al., 2011) and that they represented a localised pocket or outbreak of infection. Nevertheless, predators were still seropositive for *C. burnetii* in this area overall, albeit at a similar level to that in their prey. This indicates that the study approach of sampling carnivores may not be as useful for detecting more localised reservoirs or outbreaks of infection but is better aimed, as intended, at gaining a generalised picture of presence or absence of a pathogen in a wider area (VerCauteren et al., 2008).

The finding of a higher seroprevalence in female rodents compared to males differs from findings for other wild rodent pathogens. For example, male wood mice and bank voles have been found to have a higher infection risk for murid gammaherpesvirus 4 (Telfer et al., 2007). A UK study on cowpox in bank voles and wood mice found, from longitudinal studies, that males are more commonly infected, but there is no clear association with age, as found in the present study. The authors suggest this is applicable to other rodent-pathogen interactions (Hazel et al., 2000a) so the reasons for the different findings in seroprevalence in relation to sex in the present study are unclear.

Ejercito *et al.* (1993) used a distribution analysis approach to determine threshold

values when they used an ELISA test for a serosurvey of a variety of species of wild animals for antibodies against *C. burnetii* in Japan. As in this study they substituted a Protein A and G conjugate for species specific antibodies as a method for overcoming the lack of specific antisera to IgG for wildlife species (Gardner et al., 1996). They used a qualitative (titre) rather than quantitative (S/P) approach to the interpretation of results in the absence of known species specific thresholds. A threshold value for considering a species positive was determined by titrating serial dilutions (1:100 to 1:3,200) of serum for each animal and looking at the bimodal distribution of antibody titre per species, rather than the OD results from a single dilution per species as in the present study. However, the distribution data by which they made their cut-off decisions is not presented, so the clarity of the distinction between the two distributions is not obvious. They report that, usually, two peaks of antibody titre distribution were observed in every species with a peak in the lower dilution and a peak in the higher one, and they considered the tapering end of the first peak, which was the start of the second peak, to be the cut-off for seropositive samples. Species with only one peak at the low dilution were considered *Coxiella*-antibody negative. They found that species with a high antibody prevalence, such as black bears, deer and hares, usually had a 1:100 cut-off titre, whereas those with a low antibody prevalence such as monkeys and nutria (a rodent species) had a cut-off titre of 1:400. Webster and Macdonald (1995) used a direct IgG ELISA to test wild brown rats for antibodies to *C. burnetii* in addition to IFA testing and also used serial dilutions to determine a titre of >1:100 as positive.

In the present study, as in the studies by Ejercito *et al.* (1993) and Webster and Macdonald (1995) described above, the threshold value for being considered positive also varies between species, being determined as 15% for the three rodent prey species and 25% for cats and foxes.

Although relatively large numbers of prey species were sampled the distinction between negative and positive populations cannot be made with absolute certainty by visualising the frequency distributions of ELISA (Figure 4.6). However, the similarity of distributions in all three rodent prey species in all three areas greatly

increases confidence in the 15% threshold. Much smaller numbers of predators were sampled, especially cats (only 26), and ideally much larger numbers would need to be sampled to get greater clarity of distinct negative and positive populations and more confidence in the threshold level. Overall, this uncertainty and somewhat arbitrary nature of determination of test thresholds, and hence seroprevalence, means extrapolation of the findings of this study to the levels of exposure to *C. burnetii* that may truly be present in the population in each area is complex, but despite this potential uncertainty, the data do strongly support the principle under investigation in this study, that seroprevalence for selected pathogens will be higher in predators than in their prey.

One way of increasing confidence in the results found and interpretation of thresholds would have been to compare the results of the ELISA test used in this study with results using another serological test method such as CF or IFA. However, due to intrinsic differences between tests, interpretation can be difficult – for example the positive control in the ELISA test used was likely to be negative by CF (A LeCoq, IDVet, personal communication), because complement fixing antibodies appear later after exposure to *C burnetii*, although they persist for long periods after illness (Murphy and Field, 1970). Due to the limited amounts of serum available from the species tested in this study, use of another test was not undertaken, but testing of a subset of ELISA positive and negative samples in this way would be highly desirable. Further validation and determination of test sensitivity and specificity of the ELISA test used, by a combination of additional serological testing using another test methodology and/or direct demonstration of the presence of the pathogen, would be required to enable its future use in determining presence or absence of *C. burnetii* infection in these wild populations with confidence (see Chapter 7 for further discussion).

Although test specificity and sensitivity could not be determined in this study, the overall purpose was to investigate the patterns of seroprevalence in predators and prey, in order to find evidence to support or refute the proof of principle that carnivores can act as sentinels for certain diseases in their prey. Using the thresholds

determined, this part of the study provides good evidence to support the proof of principle. Although seroprevalence is consistently higher in predators in all study areas, levels of seroprevalence in prey and predators within and between areas do not appear to follow each other closely and no consistent positive patterns emerge.

In addition to providing evidence that carnivores can act as sentinels for *C. burnetii*, this is the first report of seroprevalence *to C. burnetii* in bank voles, field voles, wood mice, foxes and cats in the UK. In rodents, this pathogen has only been reported in wild brown rats in the UK (Webster et al., 1995b). Antibodies have been reported in cats and wild carnivores before, including foxes in the US (McQuiston and Childs, 2002), but not in the UK. The high seroprevalence in domestic cats found in this study (34.62%) confirms the potential zoonotic risk of this species (Marrie et al., 1988; Matthewman et al., 1997).

In the next chapter, a similar approach is used to investigate seroprevalence of *Leptospira* spp in predators and prey, but for this pathogen the use of a "gold standard" was also employed for a subset of samples to assist in validation of the test.

Chapter 5. *Leptospira* spp.

5.1 Introduction

The second pathogen selected to test the proof of principle that carnivores can act as sentinels for infectious diseases present in their prey was *Leptospira*. Leptospires are spirochaetes (thin, helical bacteria) belonging to the genus Leptospira in the family Leptospiraceae (Levett, 2001). Until recently, the genus was divided into two species - *Leptospira interrogans*, comprising all the pathogenic strains, and *L. biflexa*, comprising the saprophytic environmental non-pathogenic strains (Levett, 2001). Traditionally, classification is serological, based on antigenic determinants, with the basic systematic unit being the serovar. The serovar definition is based on agglutination after cross-absorption with homologous antigen, and differences between serovars are dependent on variations in the carbohydrate side chains of the lipoplysaccharide (LPS) of the cell membrane (Faine et al., 1974). Related serovars which cross-agglutinate are placed in serogroups, and under this system, *L. interrogans* has over 200 recognised serovars and *L. biflexa* over 60 (Bharti et al., 2003). However, this phenotypic, non-taxological classification has more recently been joined by a parallel genotypic system of, currently, 13 pathogenic Leptospira species (Adler and de la Pena, 2009). Unfortunately, the two systems do not overlap, meaning that serogroup or serovar do not necessarily have any reflection on the taxonomic species of *Leptospira*. Despite this more modern classification based on genomospecies, the traditional system of serogroups and serovars is more familiar to both clinicians and epidemiologists and continues to be used widely, especially for diagnostic purposes (Bharti et al., 2003).

In man *Leptospira* causes a spectrum of disease, from subclinical infection to a severe fatal multi-organ disease characterised by jaundice and renal failure, first reported by Adolf Weil in 1886 and referred to as “Weil’s disease” (Leonard et al., 1992; Levett, 2001; Vijayachari et al., 2008). Domestic animals, mainly dogs, cattle, pigs and horses, can also suffer from leptospirosis, and in these species fever, renal and hepatic insufficiency and reproductive failure can be seen (Adler and de la Pena, 2009). Host-adapted serovars in many species usually do not cause any significant

clinical signs, such as canicola in dogs, bratislava in horses and pigs, hardjo in cattle, australis and pomona in pigs, but other non host-adapted serovars can cause serious disease in animals (Adler and de la Pena, 2009). Other animal species, particularly wild rodents, have been found to harbour host-related serovars in their proximal renal tubules without any apparent clinical signs of disease and shed the organism into the environment (Thiermann, 1981), making them an important potential reservoir of infection for other animals and man. Infected domestic animals can also become asymptomatic carriers, including those recovering from leptospiral disease, and shed *Leptospira spp.* into the environment via the urine for extended periods (Harkin et al., 2003; Leonard et al., 1992; Levett, 2001). Worldwide, almost every species of mammal has been shown to be a carrier of leptospires (Adler and de la Pena, 2009).

Leptospira spp. were selected as a suitable candidate pathogen for the proof of principle under investigation in this study due to:

- their well-described rodent reservoir (Levett, 2001)
- ingestion in carnivores being recognised as a potential means of infection (Shophet and Marshall, 1980)
- recognition of leptospirosis as an important and re-emerging disease worldwide (Levett, 2001).
- reliance on serological testing as a gold standard (OIE, 2008a),

In addition, the last published survey on *Leptospira spp.* in British wild mammals was over 40 years ago (Twigg et al., 1969), and this lack of more recent or current knowledge on their presence in UK wild rodents and predators also prompted their investigation.

5.2 Test options for *Leptospira* spp.

Diagnosis of leptospirosis in man and other animals can be made by demonstration of the organism or by serological tests for the detection of leptospiral antibodies. Definitive diagnosis is by culture from blood, CSF, tissues or urine collected in the first weeks of infection, but this is very slow (a minimum of 13 weeks incubation before cultures can be deemed negative), has low sensitivity, and is not considered

useful as a diagnostic test for clinical cases in humans (Adler and de la Pena, 2009; Ahmad et al., 2005). Other direct techniques can be used, such as visualisation of leptospires using dark-field microscopy, immunofluorescence, antigen ELISA and immunoprecipitation to demonstrate the presence of leptospires in body fluids (blood, urine, milk) or tissues. All of these methods can give a definitive diagnosis of acute clinical disease, and, in animals, chronic infection of the mother if the organism is found in the foetus (OIE, 2008a). Isolation or demonstration from the kidney, urine or genital tract of animals not showing clinical signs is diagnostic only of a chronic carrier state, but are not routinely used (Adler and de la Pena, 2009; Ahmad et al., 2005). Molecular methods are more useful and several PCR protocols have been developed (e.g. (Cai et al., 2002; Cheemaa et al., 2007; Levett et al., 2005; Reitstetter, 2006; Smythe et al., 2002). However, most of these direct methods do not identify the infecting serovar, and results need to be interpreted in conjunction with serological results (OIE, 2008a).

Serological testing is the most frequently used diagnostic tool for leptospirosis, and was the selected method for this study. Available serological tests include the microscopic agglutination test (MAT) (Cole, Jr. et al., 1973), and various ELISA techniques (Adler and de la Pena, 2009). The indirect haemagglutination assay (Levett and Whittington, 1998), macroscopic agglutination (slide) test (Brandao et al., 1998), latex agglutination (Dey et al., 2007), lateral flow assays (Smits et al., 2001) and IgM dipstick (Smits et al., 2000) may also be used, but these are generally employed as rapid screening tests for acute disease in humans, whereas the MAT and ELISA tend to be employed in epidemiological studies.

In humans, the antibody response to leptospiral infection is characterised by seroconversion from days 6-10 after the onset of disease symptoms (Ahmad et al., 2005). IgM antibodies appear earlier than IgG and remain detectable for months or even years but at a low titre (World Health Organisation, 2003). Antibody levels generally peak 3-4 weeks after the onset of disease (Ahmad et al., 2005). Antibodies to LPS have been found to be predominantly IgM while antibodies to leptospiral proteins were exclusively IgG (Guerreiro et al., 2001). Seroconversion, or a four-fold

increase in antibody titre between paired serum samples, is considered diagnostic in both man and other animals. A high IgM titre in a single test is also consistent with current or recent infection. IgG may not be detected at all, or for only short periods of time, but may sometimes persist for years. In chronically infected carrier animals antibody titres may fall to undetectable levels despite continuing infection, and in these cases sensitive methods to detect the organism in urine or the genital tract may be used (OIE, 2008a). Antibodies are generally serovar-specific, but in the acute phase of infection significant cross-reaction can occur between serovars and serogroups (World Health Organisation, 2003).

5.2.1 Microscopic agglutination test (MAT)

The MAT is considered the gold standard reference method for serological diagnosis of leptospirosis in humans and other animals (OIE, 2008a; World Health Organisation, 2003). In this test, antibodies are measured by incubating serial dilutions of serum with live suspensions of a panel of leptospiral serovars. After incubation the serum/antigen mixture is examined by dark-field microscopy for the presence of agglutination of the leptospires by antibodies. The end point is the highest dilution of serum at which 50% agglutination occurs, determined by the presence of approximately 50% free, unagglutinated leptospires when compared to a control culture diluted 1:2 in phosphate-buffered saline (World Health Organisation, 2003). Because agglutinating antibodies usually only react with a certain serovar or serogroup, the MAT is used to determine which of these is likely to be causing infection. However, especially in early infection, there can be significant cross-reactivity between serogroups and serovars, and thus definitive confirmation of the infecting serovar can only be confirmed by isolation (culture) (World Health Organisation, 2003).

To perform the MAT, live cultures of leptospiral strains representing all serogroups need to be maintained by each testing laboratory, so the test cannot be completely standardised. MAT is often performed somewhat differently by different laboratories (Chappel et al., 2004); it requires significant expertise and time to perform and inter-laboratory variation in results is considered high due to its subjective interpretation

(Bharti et al., 2003; O'Keefe, 2002). Accuracy of the decision point for a positive result of 50% agglutination purely by visual observation must be questionable, and in the author's experience when performing MAT in -house was frequently difficult. Quality control measures are not always used to ensure the integrity of live reference strain panels (McBride et al., 2005), but the International Leptospirosis Society launched an initiative in 2004 to establish proficiency testing and this has been demonstrated to improve laboratory performance (Chappel et al., 2004).

The MAT detects both IgM and IgG classes of agglutinating antibodies (World Health Organisation, 2003). In acute infections in man and other animals, seroconversion or a 4-fold increase in titre between paired sera is used for diagnosis (OIE, 2008a). In clinically normal animals where a chronic carrier state is more likely, a result from a single sample of 50% agglutination at a dilution of 1/100 is often considered significant (O'Keefe, 2002). The reported sensitivity and specificity of the MAT are high in detecting human clinical leptospirosis, 92% and 95% respectively (World Health Organisation, 2003). However, the MAT may be less sensitive for detecting infections of serovars in maintenance or reservoir hosts than in non-maintenance hosts (O'Keefe, 2002), and antibody titres may fall to undetectable levels while the animal remains chronically infected (OIE, 2008a). For example, cattle infected with *L.hardjo* can have MAT titres as low as 1/10 and a test sensitivity of only 67%, or as low as 41% if the standard 1/100 cut-off is used (Ellis et al., 1986). For acute infections in humans and animals, the high specificity and ability to identify the likely infecting serogroup/serovar are the reasons that the MAT is still considered the gold standard serological test. In animals, the MAT is also widely used as a herd test and for epidemiological studies, despite its lower sensitivity in these situations. For herds, it is recommended that at least ten animals, or 10% of the herd are tested (Cole et al., 1980).

The non species-specific nature of the MAT means that it is widely employed for serological studies of wildlife (Akerstedt et al., 2010; Diesch et al., 1970; Ferguson and Heidt, 1981; Fleming et al., 1979; Khan et al., 1991; Kingscote, 1986; Kositanont et al., 2003; Lilenbaum et al., 2004; Matthias and Levett, 2002; Millan et

al., 2009; Richardson and Gauthier, 2003; Rim et al., 1993; Slavica et al., 2010). However, the possibility exists that antibodies may not be detected if the causative strain is not represented in the test or only a low titre is found with a serovar that antigenically resembles an absent causative serovar. Similarly, new, unidentified serovars may be causing disease, and it is never possible to be sure that a MAT panel is complete, allowing false negatives to occur. The MAT is also expensive when performed commercially (e.g. £27.45 plus VAT for initial screen, then £25.80 plus VAT for specific pools, then a further £15.20 for specific serovar, VLA Weybridge).

Largely for these reasons, screening tests based on genus-specific broadly reactive antigens, have been developed, in particular the ELISA (see 5.2.2). The ELISA is also more suitable for epidemiological studies, such as the present one, where one aim is to detect chronic enzootic infection in reservoir hosts with associated low antibody titres. Although many ELISA tests are only genus specific, and cannot identify serogroup or serovar, they have the advantage of being able to detect anti-leptospiral IgM antibodies before agglutinating antibodies appear, and thus earlier than the MAT (Cumberland et al., 1999).

In this study, a novel ELISA was therefore developed that could be applied to multiple and non -domestic species. In addition, due to the labour-intensive nature of the MAT and its cost, which was prohibitive for the large number of wildlife samples used in this study, the development of a novel ELISA was also pursued as a more time and cost-effective approach for the proof of principle. The MAT was, however, used on a subset of samples (24.4%) to compare results with the ELISA and to see if any conclusions could be drawn about the applicability of either test in investigation of the proof of principle.

5.2.2. ELISA

ELISA tests for *Leptospira spp*. have been developed using a wide variety of antigen preparations, and avoid the need for maintenance of live cultures, with the attendant biosafety requirements (level 2). The ELISA can also be automated, standardised and prepared in kit or dipstick form for rapid use in clinical situations. Whole cell

leptospira antigen- based ELISA tests have been commercially developed in an attempt to improve serologic diagnosis in humans and many commercial kits have been evaluated in large human studies. These ELISAs have been found to have sensitivities ranging from 28 – 72% and specificities from as low as 10% up to 99% (McBride et al., 2005; McBride et al., 2007). ELISAs used in man and domestic animals are most commonly used for detection of anti-leptospiral IgM antibodies, which become detectable during the first week of illness (Adler et al., 1980; Bajani et al., 2003; Bharti et al., 2003; Hartman et al., 1984a; Hartman et al., 1984b; Levett, 2001).

However, in domestic livestock and dogs ELISAs that measure IgG have also been found useful in epidemiological and vaccination studies as these antibodies persist longer (Cousins et al., 1985; Cousins et al., 1991; Cousins and Robertson, 1986; Hartman et al., 1986). In domestic animals, some serovar- specific ELISAs have been developed, for example for the detection of serovars pomona and hardjo in cattle and sheep (Adler et al., 1981; Thiermann and Garrett, 1983).

Leptospiral ELISAs used in man and animals employ species –specific antisera, and thus are only applicable to the species for which this antisera is available. In studies on wild species where species- specific antibodies are not available, either the closest related animal antisera has been used, on the assumption that inter-species reactivity will occur, e.g. a combination of anti-rat and anti-hamster IgG in wild rodents (Vanasco et al., 2001), or, more commonly, the MAT has been employed to circumvent this issue.

The various ELISA antigen preparations used include whole formalin-killed leptospires (World Health Organisation, 2003) and whole cell lysates (Bercovich et al., 1990; Ribotta et al., 2000) (Vanasco et al., 2001). The non-pathogenic *L. biflexa* can be used as the basis of a genus-specific ELISA as there is cross-reactivity between this and *L. interrogans* serovars (Hartman et al., 1984c; World Health Organisation, 2003). However, some of the outer membrane structural and functional proteins and lipoproteins have been shown to be antigenic (Adler and de la

Pena, 2009; Biswas et al., 2005) and ELISA antigens used experimemtally also include outer sheath protein (Cho et al., 1989) and recombinant cell-surface lipoproteins (Flannery et al., 2001). Previous studies have demonstrated that protein extraction of various *Leptospira* serovars by a variety of methods results in production of common immunoreactive proteins that are shared among the strains studied (Biswas et al., 2005; Brown et al., 1991; Nicholson and Prescott, 1993). The most prominent leptospiral protein in these extracts has been identified as the major outer membrane protein (MOMP) LipL32, which is expressed during mammalian infection (Haake et al., 2000).

These studies indicated that use of these common proteins as antigens would help to develop diagnostic kits (Biswas et al., 2005) and new ELISAs have been developed based on recombinent LipL32 and other proteins in both human and animal diagnostics, but are not yet widely available (Bomfim et al., 2005; Dey et al., 2004; Mariya et al., 2006; Okuda et al., 2005; Qiu et al., 2008). Leptospiral common proteins are also of interest as a basis of alternative vaccine strategies (McBride et al., 2005) as most animal vaccines are based on the LPS-carbohydrate serovar determinant and do not provide cross-protection.

Although some newer ELISAs employ extracted leptospiral proteins as antigens, there is evidence that the main antigens and serovar specific determinants are in fact the outer membrane lipopolysaccharides (LPS) and lipid antigens (Cho et al., 1992; Masuzawa et al., 1990; Shimizu et al., 1987). For example, monoclonal antibodies produced against leptospiral LPS were found to have no cross-reactivity with serovars for heterologous serogroups (i.e. were serogroup specific) (Yan et al., 1999). Anti-LPS agglutinating antibodies have also been demonstrated to provide protection against leptospiral infection (Adler and de la Pena, 2009; Jost et al., 1986).

Antibodies to LPS have been found to be predominantly IgM while antibodies to leptospiral proteins were found to be exclusively IgG (Guerreiro et al., 2001). This may impact on direct comparison of results based on ELISAs using leptospiral protein antigens (either IgG or IgM detecting) with those using the MAT, as serovar

determination by the MAT may therefore rely mainly on LPS-detecting IgM agglutinating antibodies rather than IgG. It also may mean that ELISA testing relying on protein antigens may not be useful for serovar determination.

In this study, detection of IgG as an indicator of chronic infection or exposure in healthy predator and reservoir prey species was the main focus of the study for seroprevalence purposes, and therefore extraction of leptospiral protein antigens was used. IgG ELISA kits are available specifically for testing human serum, based on extracts of *L. biflexa*, (e.g. Leptospira IgG ELISA kit, Diagnostic Automation/Cortez Diagnostics Inc., Calabasa, California) and reliant on cross-reaction between this and pathogenic Leptospira species. However, the only commercially available laboratory based ELISA tests for animals in the UK are serovar and animal species specific, e.g *L.hardjo* antibodies for cattle (VLA, Weybridge). ELISA kits for animals are available, but are again species specific and detect a limited range of serovars relevant to that species only, e.g the SmartVet Comb Canine Leptospira Antibody Test Kit ™ (Orgenics Ltd., Yavne, Israel) for detection of antibodies to *L.icterhaemorrhagiae* (*L.copenhageni* and RGA), *L.canicola, L.pomona* and *L. grippotyphosa* in domestic dogs. Due to these limitations the approach taken in this study was to develop a novel ELISA based on as wide a range of serovars as possible and applicable to multiple species including wild species for which there are no available species-specific antibodies

5.3 Materials and methods

5.3.1 Development of an indirect ELISA for leptospiral antibodies

Fifteen type cultures of Leptospira strains were obtained from the Leptospira Reference Unit (Health Protection Agency (HPA), Hereford, UK) for use as the antigen source for the ELISA. These strains represented 15 of the 16 serogroups recommended by the WHO to be included in a MAT panel. The sixteenth, serogroup Semaranga, was not available. The type cultures used are detailed in Table 5.1. Reagents used are given in Appendix 5.1

Cultures were maintained in EMJH (Ellinghausen and McCullough, modified by

Johnson and Harris) medium and subcultured weekly. Cultures were maintained in the dark at 30°C and were checked regularly for contamination and viability (density and motility) using dark-field microscopy, according to standard protocol described by the WHO (World Health Organisation, 2003).

Table 5.1 Reference type cultures of *Leptospira* maintained by live culture and used as the basis for the ELISA

Strain No.	HPA Ref Strain No.	Serogroup	Serovar	Strain
1	3	Australis	bratislava	Jez Bratislava
2	39	Bataviae	bataviae	Van Tienen
3	33	Ballum	castellonis	Castellon 3
4	130	Javanica	poi	Poi
5	175	Pyrogenes	pyrogenes	Salinem
6	108	Icterohaemorrrhagiae	icterhaemorrhagiae	Ictero 1
7	89	Hebdomanis	hebdomanis	Hebdomanis
8	85	Grippotyphosa	valbuzzi	Balbucí
9	15	Autumnalis	autumnalis	Akiyami A
10	160	Pomona	pomona	Pomona
11	69	Cynopteri	cynopteri	3522 C
12	195	Sejroe	hardjo	Hardjoprajitno
13	53	Canicola	canicola	Hond Utrecht IV
14	234	Tarassovi	tarassovi	Perepelicin
15	110	Icterohaemorrhagiae	icterohaemorrhagiae	RGA

Leptospiral protein extraction

Extraction of immunoreactive proteins from each leptospiral reference culture was attempted initially using detergent lysis, but this was unsuccessful. Successful protein extraction, confirmed by protein estimation assay, was achieved using urea and the resultant extract used as the antigen coating of the ELISA wells. Full details of the protein extraction process are in Appendix 5.2.

ELISA validation

Validation step 1. In order to confirm that the leptospiral extract was antigenic it was

initially tested against serial dilutions of reference antisera (see appendix 5.1.5). Volumes of each of the 15 leptospiral extracts containing 1 µg protein were combined together and made up to a total volume of 5.5ml with carbonate buffer. Wells of a 96-well ELISA plate were coated overnight at 4°C with 50 µl of this mixture. Buffer solutions were made as detailed in Appendix 5 (ELISA reagents). The conjugate solution was made using 10ml dilution buffer, 1 µl Protein A labelled with horseradish peroxidase (Protein A Peroxidase Conjugate, Calbiochem, San Diego, California, USA), and 5 µl Protein G labelled with horseradish peroxidase (Protein G horseradish peroxidase, Molecular Probes, Eugene, Oregon, USA).

96-well ELISA plates were incubated with 200 µl/well of blocking buffer for 30 minutes. Blocking buffer was removed and 50 µl of reference antisera (diluted 1:10, 1:100, 1:1000,1:10,000 in blocking buffer) added in duplicate to each column of the plate (see figure 5. 1 for template). Antisera to strains 5, 6, 10, 13 were not available

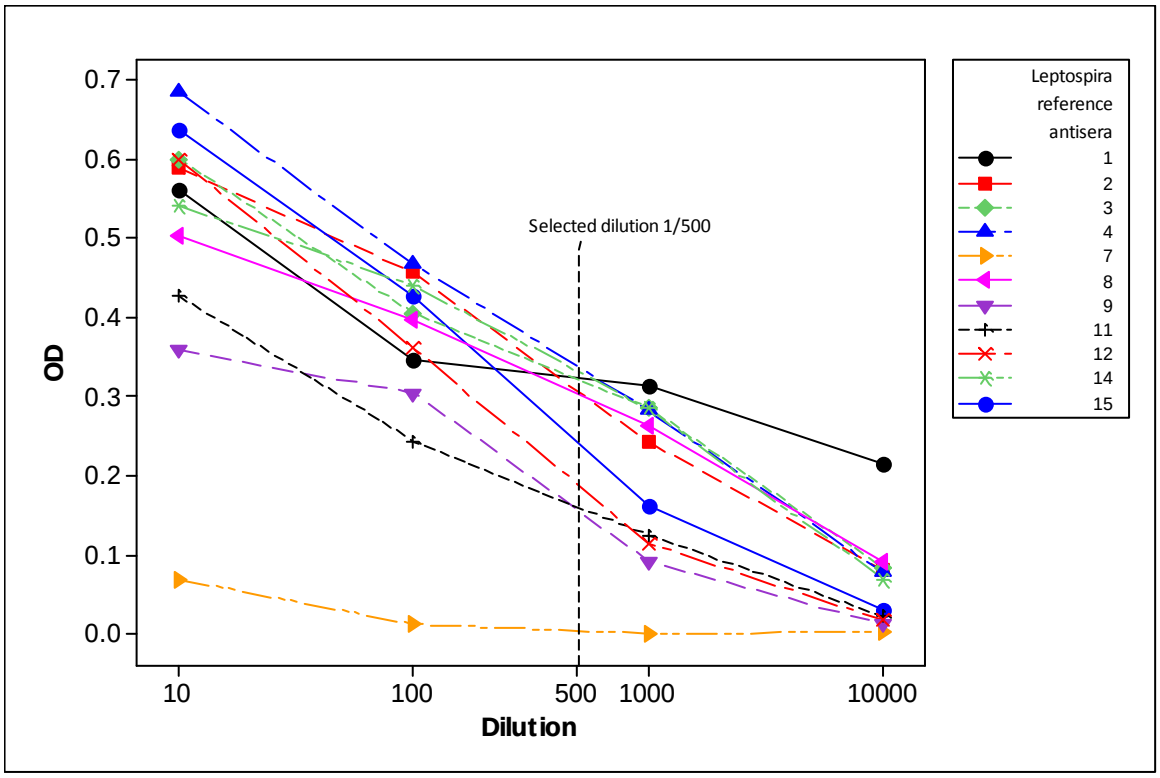
Figure 5.1 Plate template for serial dilutions of reference leptospiral antisera. All wells were coated with mixed leptospiral extract.

Antisera	1	2	3	4	7	8	9	11	12	14	15
Blank	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10
Blank	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10
Blank	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100
Blank	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100
Blank	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000
Blank	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000
Blank	1/10000	1/10000	1/10000	1/10000	1/10000	1/10000	1/10000	1/10000	1/10000	1/10000	1/10000
Blank	1/10000	1/10000	1/10000	1/10000	1/10000	1/10000	1/10000	1/10000	1/10000	1/10000	1/10000

Plates were sealed and incubated for 60 minutes at room temperature. The plate was washed 6 times with wash buffer and incubated for 60 minutes at RT with 50 µl/well of Protein A/G conjugate. The plate was washed 6 times with wash buffer and incubated for 5 minutes at RT with 50 µl/well of SureBlue TMB Microwell Peroxidase Substrate (KPL, town, country). The reaction was stopped after 5 minutes by the addition of 50µl/well of 0.18mM H2SO4 and absorption was measured at 450

nm using a Microplate Reader (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK). 1/500 was selected as a suitable dilution for further validation steps (Figure 5.2) as at this dilution all the antisera, except to strain 7, resulted in sufficient optical densities to enable efficient use of the limited quantities of antiserum available.

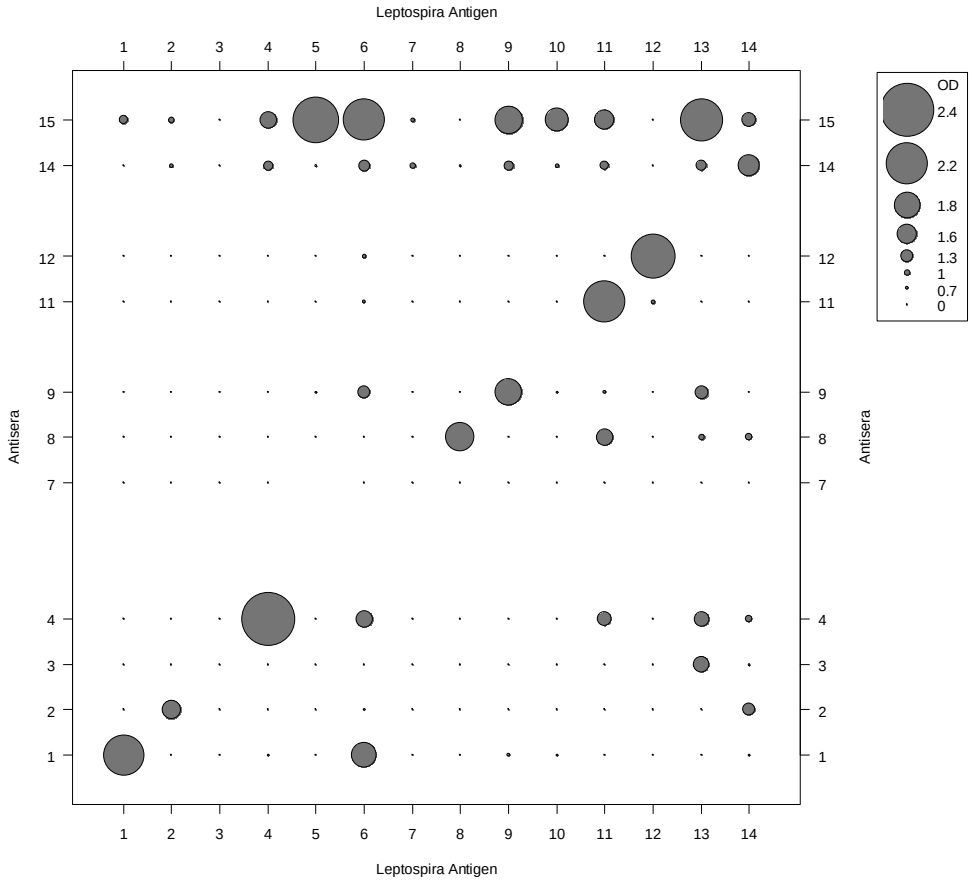
Figure 5.2 Optical densities of 11 reference leptospiral antiserum at dilutions of 1:10, 1:100, 1:1000, 1:10,000 tested by ELISA against the extracted leptospiral antigen mix.



Validation step 2. The ELISA was then performed to assess the response of the antigen preparation of each individual leptospiral strain against each reference anti-serum. Antisera 5, 6 and 10 were not obtainable from the reference laboratory, and insufficent antigen from strain 15 was available for this test but the same serogroup and serovar was represented in strain 6. Two rows of four 96-well ELISA plates were coated with 50 µl/well of each individual leptospiral serovar urea soluble extract, incubated overnight, and blocked as before. 50 µl/well of a 1:500 dilution of the corresponding antiserum was then added, and the ELISA protocol continued as

described above (Figure 5.3).

Figure 5.3 Plot of optical density of reference leptospiral antisera 1,2,3,4,7,8,9,11,12,14, and 15 to individual extracts of leptospira cultures 1 to 14. The diameter of the symbol is proportionate to the optical density of the ELISA test result



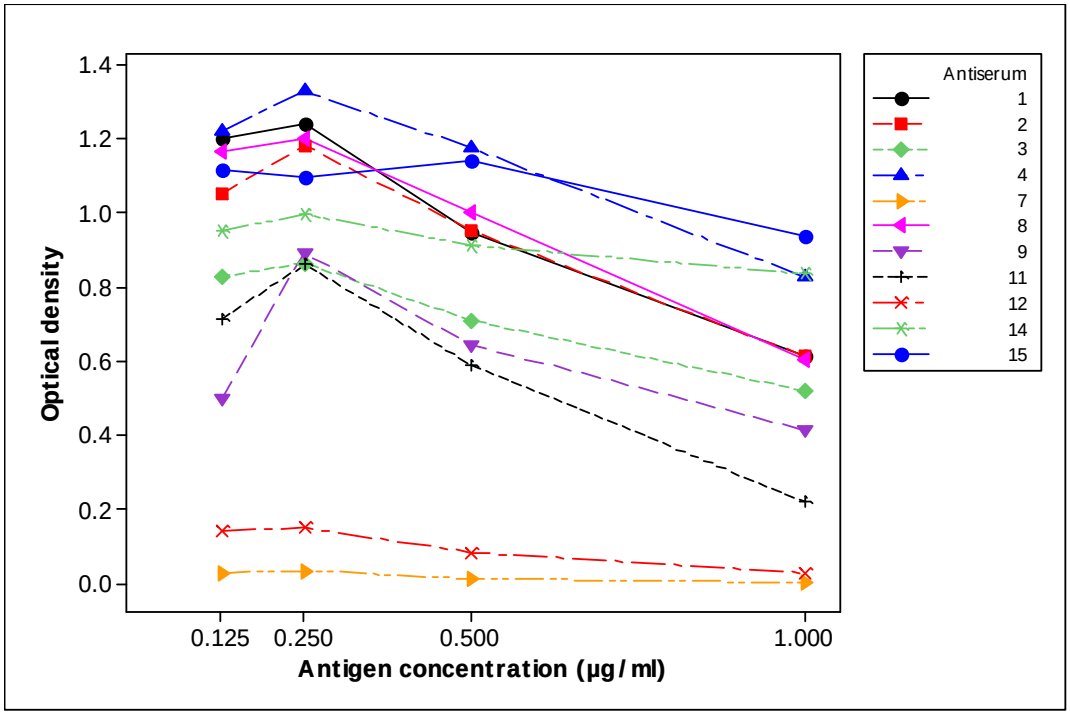
Seven extracts (from leptospiral cultures 1, 2, 4, 8, 11, 12 and 14) bound most strongly with their corresponding antisera. Extracts from cultures 3 and 7 did not bind significantly with antisera 3 and 7 or any other antisera. Extracts from cultures 5, 6, 10 and 13 bound most strongly to antiserum 15. Extract from cultures 13 bound most strongly to antisera 15.

Validation step 3. Having determined successful antigen-antibody binding, the optimum concentration of the mixed leptospiral antigen solution for coating the ELISA plates, was determined. A second batch of cell lysates, obtained by urea/ FastPrep® extraction as described above was prepared from all 15 leptospiral strains,

followed by purification and protein estimation, and SDS-PAGE performed to verify protein extraction. Using the antigen solutions from each batch with the highest protein concentration, a final single batch of mixed leptospiral antigen solution was made by mixing together volumes of each solution to make a mixture with a concentration of 1 µg/ml of each protein. The resultant mixture was divided into aliquots and stored frozen at -70°C. To determine the optimal coating concentration, 1 µg/ml; 0.5 µg/ml; 0.25 µg/ml and 0.125 µg/ml of the mixed leptospiral antigen solution were used to coat duplicate rows of a 96-well ELISA plate, and 1:500 dilutions of each of the 11 reference antisera assayed in each column.

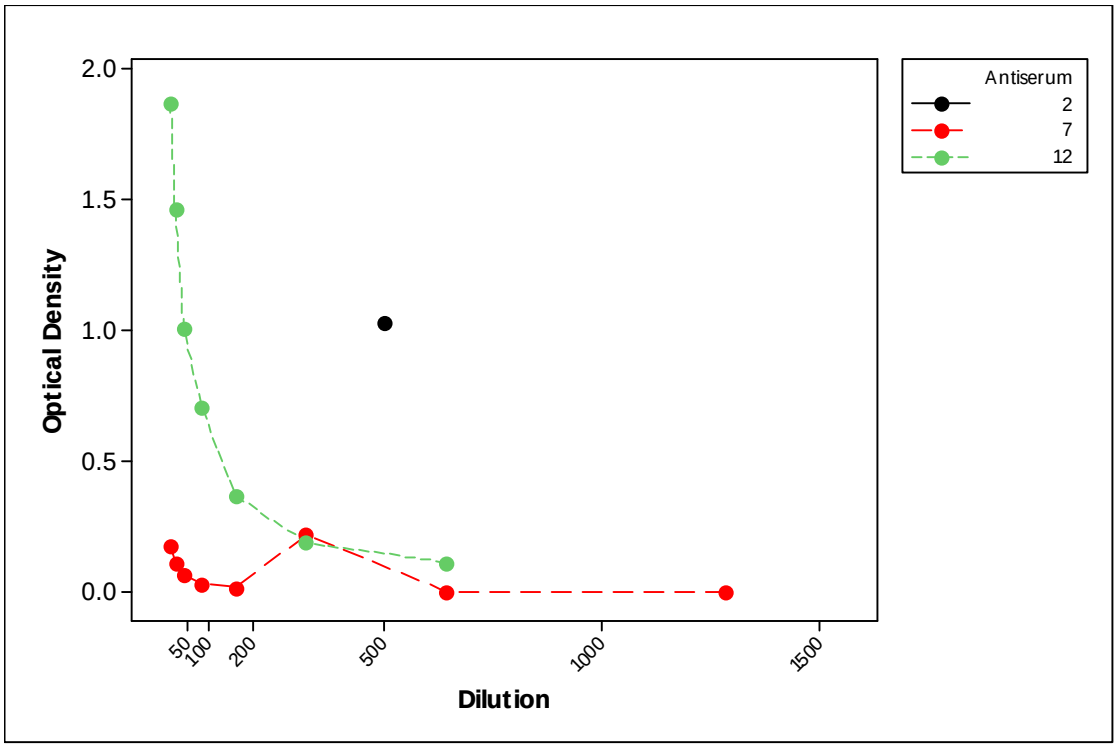
All test wells were positive with high optical density to the lower concentrations, with the exception of antisera 7 and 12, but OD decreased above antigen concentrations of 0.25 µg/ml (Figure 5.4) As the majority of optical densities were so high, a solution containing 0.1 µg/ml, slightly less than the lowest concentration tested, of each protein extract was selected as the suitable coating solution for the ELISA as it was considered that this would be sufficient.

Figure 5.4 Optical densities obtained by ELISA of 1:500 dilutions of reference antisera against four different concentrations of mixed leptospiral antigen solution.



Validation step 4. In order to determine the optimum dilution of the test sera, serial dilutions of two reference sera (7 and 12) were tested using the ELISA protocol. They were selected as they gave the weakest results in validation step 3 as described above when tested against the mixed antigen, and were thus felt to be most representative of test sera collected from animals for the study. A single dilution (1:500) of reference serum 2 was also used as a control as this was known, from validation step 1, to give a high optical density reading. Results are summarised in Figure 5.5. A dilution of 1:50 was determined as a suitable test serum dilution.

Figure 5.5 Optical densities obtained by ELISA of serial dilutions of reference antisera 7 and 12 with antisera 2 as control, against 0.1 µg/ml leptospiral antigen mix.



Following this validation phase, the test protocol of the final ELISA was determined as follows, and is detailed in Appendix 5.1.7.

- Test serum samples were diluted to 1:50 in dilution buffer and tested in duplicate.
- Controls run on every sample test plate included eight blank wells, two

human positive controls (1:50) and two negative controls of normal mouse serum (1:50).

- The test conjugate consisted of horseradish peroxidase labelled purified protein A (1mg/ml, Calbiochem) and purified protein G (1mg/ml, Molecular Probes) diluted at 1:10,000 and 1:2,000 respectively in dilution buffer.
- The substrate used was SureBlue TMB Microwell Peroxidase Substrate (1-Component)(KPL), and 0.18mM H2SO4 was used to stop substrate colour development immediately before the optical densities were read at 450nm.

For each sample the raw optical density (OD) results were normalised by adjusting each sample to the mean values obtained for the positive and negative controls, and the S/P value calculated:

$$S/P = \frac{OD_{sample} - OD_{nc}}{OD_{pc} - OD_{nc}} \times 100$$

5.3.2 MAT

A subset of serum samples were submitted to the VeterInary Laboratories Agency (VLA), Weybridge for testing using the MAT. Where possible samples with ELISA test results of S/P > 5%, and a selection of ELISA samples S/P <5% were submitted. The 5% level was used in an initial decision making process based only on S/P results before thresholds for each species had been determined (see 5.4.2). A further subset of ELISA S/P >5%, MAT negative and ELISA S/P>5%, MAT positive samples were then resubmitted blinded for retesting by MAT. Samples were tested in accordance with the method outlined in section 5.2.1 above in adherence with the OIE protocol (OIE, 2008a). Samples were filtered on receipt to remove bacterial and other contaminants. The VLA protocol used a panel of 19 Leptospira strains grouped into six antigen pools. Samples were tested against all six antigen pools (multiple serovar screening test). Any sample for which any degree of agglutination was observed against any of the pooled antigens was then tested against the individual constituent serovars in that pool with a starting sera dilution of 1:100. The pools were:

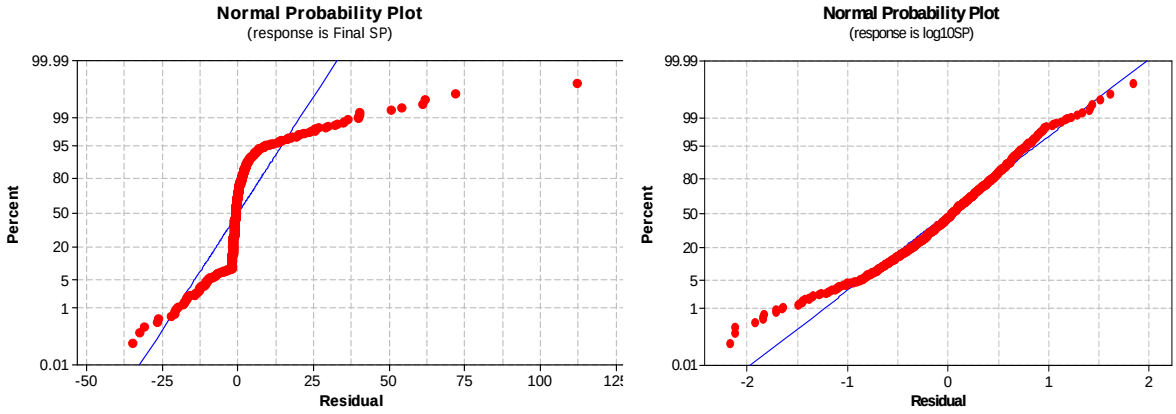
- Leptospira Pool 1 (*L. canicola,copenhag, ballum, icterohaemorrhagiae*)
- Leptospira Pool 2 (*L. pomona, mozdok,tarassovi, grippotyphosa*)
- Leptospira Pool 3 (*L. australis,bratislava, autumnalis*)
- Leptospira Pool 4 (*L. hebdomanis, mini, sejroe*)
- Leptospira Pool 5 (*L. javanica, bataviae,zanoni*)
- Leptospira Pool 6 (*L. hardjo* serovars hardjoprajitno and hardjo bovis B215)

5.3.3 Data analysis

Statistical analysis of the results was performed using Minitab 15® and R® (R Foundation). Although the intention was to use general linear mixed effect modelling on the raw S/P values, normality of the residuals was not achieved (Figure 5.6).

Figure 5.6 Probability plots for residuals for (a) raw S/P values and (b) log 10 S/P values

a) **b)**



Kruskal-Wallis analysis was therefore used to test for differences in S/P values between predator and prey species overall, by individual species and by study area. Post hoc analysis of all pairwise comparisons (Dwass-Steel-Critchlow-Fligner method) (Hollander and Wolfe D.A, 1999) was used for differences in S/P values between predator and prey species was performed using StatsDirect® Version 2.7.8 (StatsDirect Ltd). Summary variables of the S/P results (mean, median, minimum, maximum, Q1, Q3) were explored for relationships between prey and predator species, and Pearson's correlation coefficient (r) used to see if any associations were

statistically significant.

For seroprevalence data, generalised linear mixed effect modelling with binomial errors was used to explore seroprevalence in prey and predator species, with study area as a random effect for all species, and study site nested within study area for prey species. Fixed effects of sex, age, and season were incorporated. For age, animals were classed as either adult or non-adult (juvenile and subadult). For all tests used, the significance level was placed at $P<0.05$.

For comparisons of ELISA with MAT results, sensitivity and specificity were calculated and receiver operating characteristic (ROC) curves plotted.

5.4 Results

A total of 952 serum samples were tested for antibodies to *Leptospira spp*., from 820 prey species (189 bank voles, 315 field voles, 316 wood mice) and 132 predator species (26 cats, 106 foxes) (Table 5.2) . This represented 89.9 % of the total number of samples of these five species that were collected during the study. The majority of samples for both prey and predators were collected in seasons 1 and 2, and in season 4 only the Cumbria study area was sampled for prey species.

Table 5.2 Numbers of serum samples tested for *Leptospira spp*. by area, species, predator or prey status and season

Area	Predators	Total tested	Season 1	Season 2	Season 3	Season 4	Non seasonal
Borders	Cat	10					10
	Fox	40	25	4	6	5	
Cumbria	Cat	9					9
	Fox	48	1	24	10	13	
Pentlands	Cat	7					7
	Fox	18	10	2	2	4	
		132	**36**	**30**	**18**	**22**	**26**
	Prey						
Borders	Bank vole	19	5	14	0		
	Field vole	99	46	45	8		
	Wood mouse	57	22	3	32		
Cumbria	Bank vole	64	11	27	20	6	
	Field vole	115	27	46	20	22	
	Wood mouse	104	43	47	10	4	
Pentlands	Bank vole	106	46	48	12		
	Field vole	101	20	55	26		
	Wood mouse	155	82	35	38		
		820	**302**	**320**	**166**	**32**	
TOTAL		**952**	**338**	**350**	**184**	**54**	**26**

5.4.1 Raw S/P values

The raw data, expressed as sample:positive control (S/P) % was initially explored without consideration as to whether these values represented a positive or negative result. Median S/P values were significantly higher (P<0.001) in predtaors than in prey for both the study overall and in each study area (Figure 5.7 and Table 5.3).

Figure 5.7 Dot plots of raw S/P values by species and study area. Horizontal bar indicates the median value for each species in each study area. These dot plots have been jigged so that width of the "blob" of overlying dots reflects, but is not an exact representation of, the numbers of animals with this S/P value.

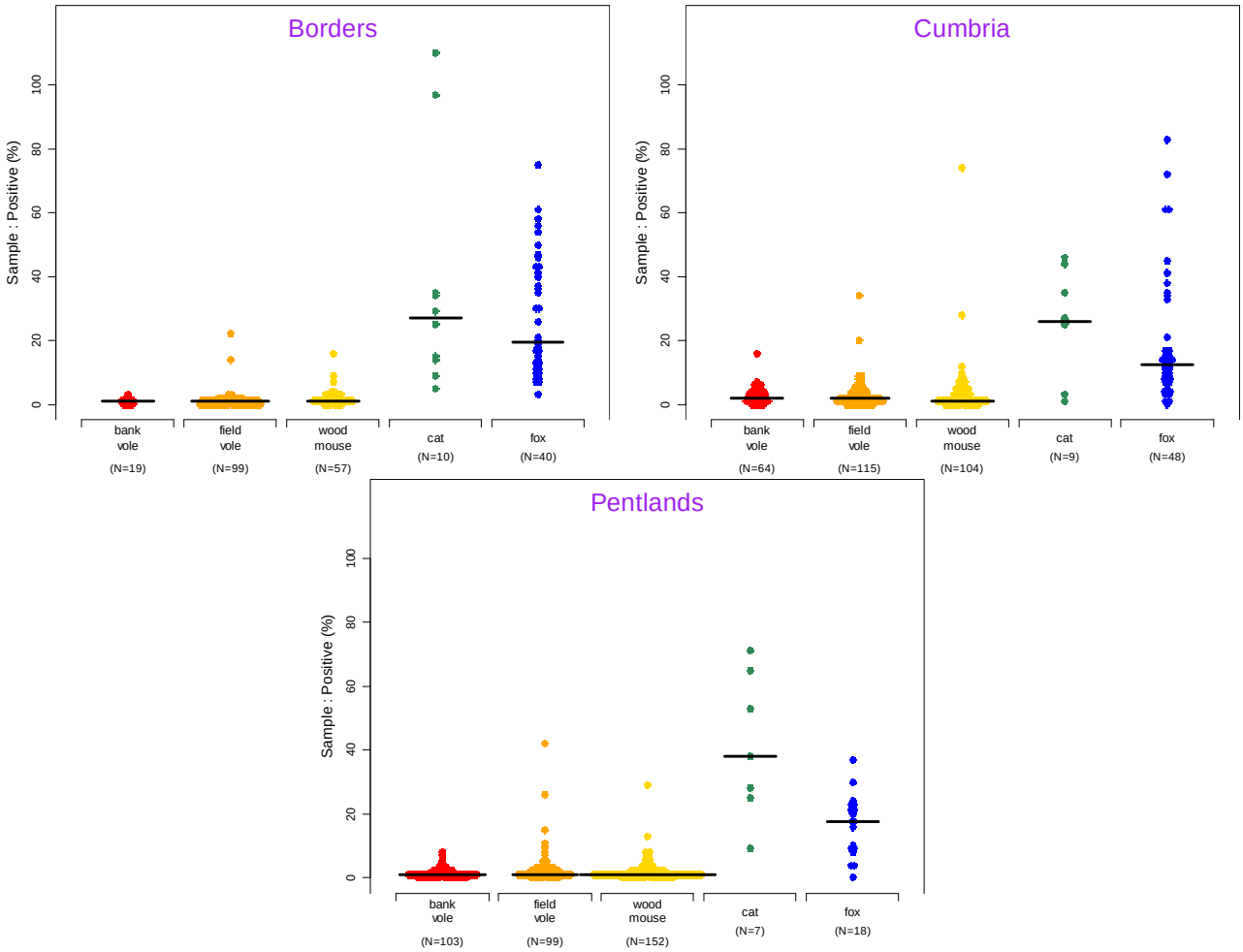


Table 5.3 Median S/P % (with ranges) and mean (with SE mean) values for *Leptospira spp*. antibodies by ELISA testing in prey and predator species overall by study area

Predator/ Prey	Median S/P	Mean S/P
	Overall	
Prey	1.14 (0.00-74.20)	2.12 (0.15)
Predator	16.78 (0.17-147.76)	24.16 (1.92)
	Borders	
Prey	0.89 9 (0-22.50)	1.40 (0.188)
Predator	20.69 (2.87-147.76)	29.85 (3.72)
	Cumbria	
Prey	1.77 (0.02-74.20)	2.94 (0.33)
Predator	13.8 (0.17-83.02)	19.49 (2.45)
	Pentlands	
Prey	1.00 (0.01-42.42)	1.82 (0.18)
Predator	21.32 (0.21-70.53)	23.4 (3.61)

At an individual species level for the study overall, median S/P values were remarkably similar at 1.00 – 1.18 for all three prey species (Table 5.4) There were no significant differences between cats and foxes (P=0.078) or between any of the three rodent prey species (P>0.90), but there were significant differences between predator species (cat and fox) and all prey species (P<0.001). This pattern of a significantly higher seroprevalence in predator than in prey species (P<0.001) was also consistent in each study area.

Study area did have a significant effect (P<0.001), with S/P significantly higher in predators and lower in prey in Cumbria compared to Borders and Pentlands, but qualitatively overall the pattern between predators and prey was the same in each area (Table 5.4 and Figure 5.7).

Table 5.4 Median S/P % (with ranges) and mean (with SE mean) values for *Leptospira spp*. antibodies by ELISA testing in individual species

Predator/ Prey	Species	Median S/P	Mean S/P
		Overall	
Prey	Bank vole	1.00 (0.01-15.74)	1.67 (0.14)
	Field vole	1.18 (0 - 42.42)	2.25 (0.23)
	Wood Mouse	1.14 (0.01-74.20)	2.24 (0.28)
Predator	Cat	27.70 (14.67-147.76)	35.83 (6.22)
	Fox	14.49 (0.17-83.02)	21.29 (1.75)
		Borders	
Prey	Bank vole	0.62 (0.07-2.99)	0.92 (0.18)
	Field vole	0.63 (0-22.50)	1.19 (0.26)
	Wood Mouse	1.14 (0.10-16.39)	1.95 (0.34)
Predator	Cat	27.20 (4.70-147.76)	41.0 (14.4)
	Fox	19.36 (2.87-75.29)	27.07 (2.95)
		Cumbria	
Prey	Bank vole	2.14 (0.08-15.74)	2.68 (0.30)
	Field vole	1.80 (0.02-34.04)	2.77 (0.52)
	Wood Mouse	1.38 (0.07-74.20)	3.29 (0.77)
Predator	Cat	25.8 (0.84-46.01)	25.79 (5.22)
	Fox	12.38 (0.17-83.02)	18.31 (2.72)
		Pentlands	
Prey	Bank vole	0.84 (0.01-8.46)	1.20 (0.13)
	Field vole	1.38 (0.02-42.42)	2.72 (0.52)
	Wood Mouse	0.98 - 0.01-28.89)	1.65 (0.22)
Predator	Cat	38.19 (9.40-7-.53)	8.50 (22.50)
	Fox	17.39 (0.21-37.49)	16.40 (2.31)

As for *C. burnetii* (chapter 4), exploration of the raw data for possible relationships between summary variables for prey and predator species in each study area (mean, median, minimum, maximum, Q1, Q3) did not reveal any obvious patterns in the raw data.

Season and lagged season

Relationships between summary variables of S/P values in foxes and prey species were also explored in terms of and lagged season (Cumbria only), as for *C.burnetii* (Chapter 4), but no consistent pattern or relationship was found (data not shown). Cats were not examined in this way because they were not allocated a sampling season.

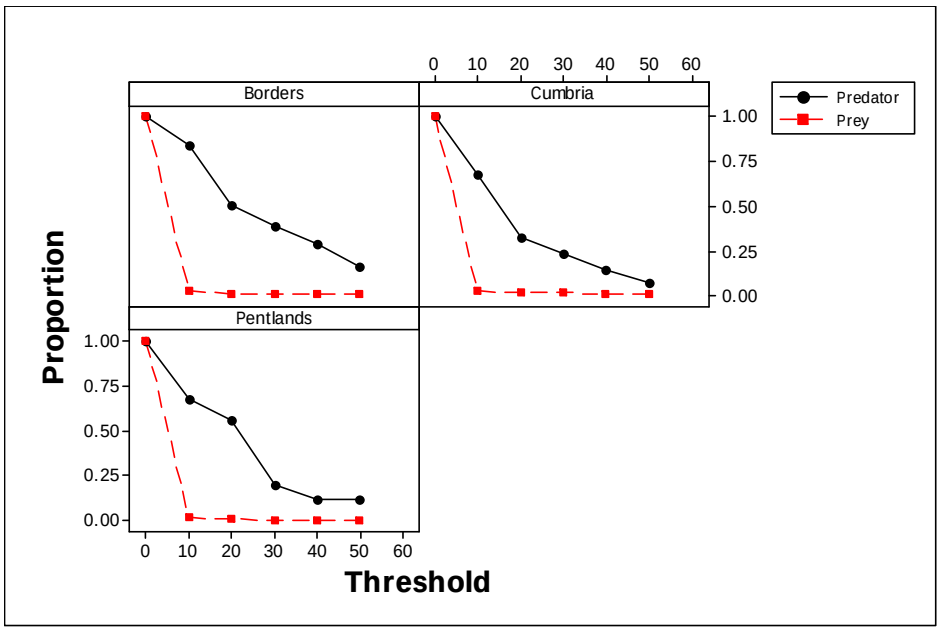
Summary of raw data results

On examination of the summary variables for the raw S/P data it was found that median values were significantly higher in predators than prey in all areas consistently and irrespective of species. Median S/P values did not differ significantly between individual predator species (cats and foxes) or individual prey species (voles and wood mice). However, no consistent positive patterns were found relating prey and predator S/P values by area, season or lagged season.

5.4.2 Thresholds

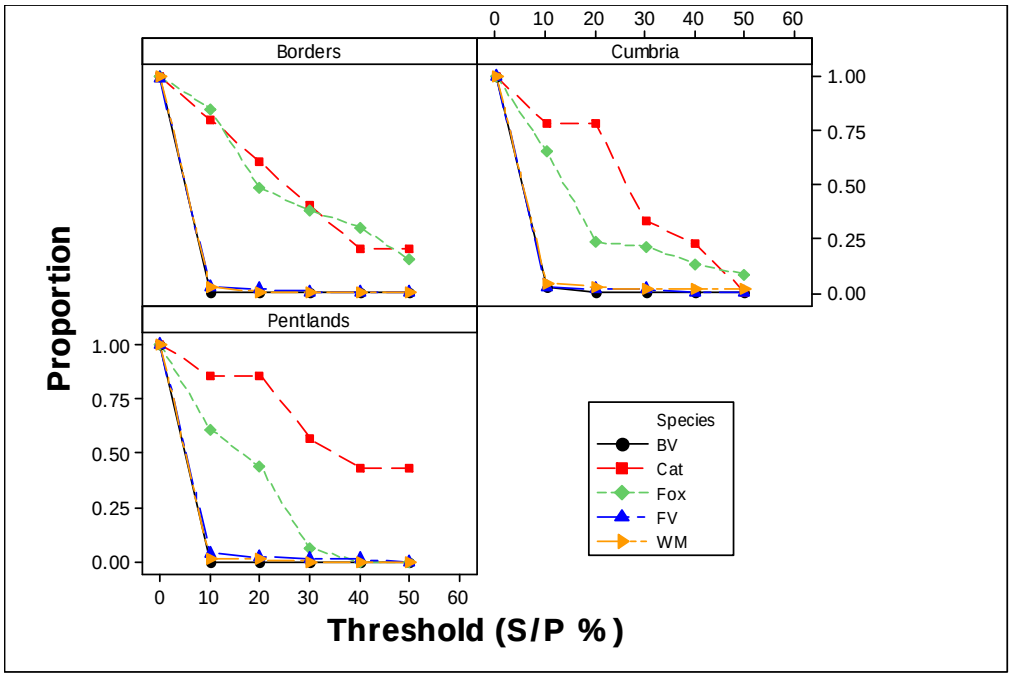
The main purpose of developing the ELISA test for use in this particular study was to use the OD and S/P results to generate information on seroprevalence of antibodies to *Leptospira spp*. in the prey and predator species investigated. As in the previous chapter with the ELISA test results for *C.burnetii*, in the absence of known positive and negative populations for each of the five species tested, thresholds were firstly explored in a simple fashion exploring various thresholds (10%, 20%, 30%, 40% and 50%) to look at proportions of prey and predator species at each, and to explore possible relationships between predators and prey at differing threshold levels. This clearly found that at each threshold the predator species have a higher proportion of animals above that particular threshold in all study areas for all thresholds up to 50% (Figure 5.8).

Figure 5.8 Proportions of predator and prey species above increasing S/P thresholds up to 50% in each study area



This pattern is also consistent for individual predator and prey species in each study area (Figure 5.9), although in the Pentlands the proportion of foxes drops to that of prey above a threshold of 30%.

Figure 5.9 Proportion of each individual species above increasing S/P thresholds in each study area (BV = bank vole, FV = field vole, WM = wood mouse).



The effect of season on the proportion of each species at or above each threshold was also explored, to see if the proportion of animals above a particular threshold might be influenced by the proportion in the preceding season. However, no consistent patterns were apparent (data not shown).

Determination of threshold from frequency distributions

As for *C. burnetii* as described in chapter 4, a more conventional approach of evaluating S/P data is by determining if the distributions fall into two distinct populations representing those with background reactivity to the ELISA test (negative) and those with specific antibodies to *Leptospira spp*. (positive).

Examination of the frequency distributions of the S/P data for the three rodent prey species shows that all three have a peak response at less than 10%, which indicates

that this is the negative population (Figure 5.10). A second distinct population is less clear than that found for *C. burnetii* as very few samples had values over 10%. However, from assessment of these frequency distributions a threshold for prey species was placed at 10% as this was judged to divide the two populations.

For the predator species (Figure 5.11), the distribution into two possible populations is less distinct. In the cats, where sample size is low (n= 26), the peak frequency is at 30%, with 4 individuals with S/P values of 65%, 71%, 97%, and 147%. If these are considered as positive samples the threshold could be placed at 60%. For the foxes, where sample size is larger (n= 106), there appears to be a second distribution above 30% and thus 30% was explored as the test threshold for this species. However, as for the rodents, a threshold of 60% was also explored for the foxes to account for the possibility that only the three foxes above this threshold might have been the true positives.

Thus in summary, the test cut-off, or threshold, was determined as:

- 10% for prey, 30% for foxes, 60% for cats

Figure 5.10 Distribution of S/P values for prey species. Dotted line at 10% indicates the selected threshold that appears to separate two populations

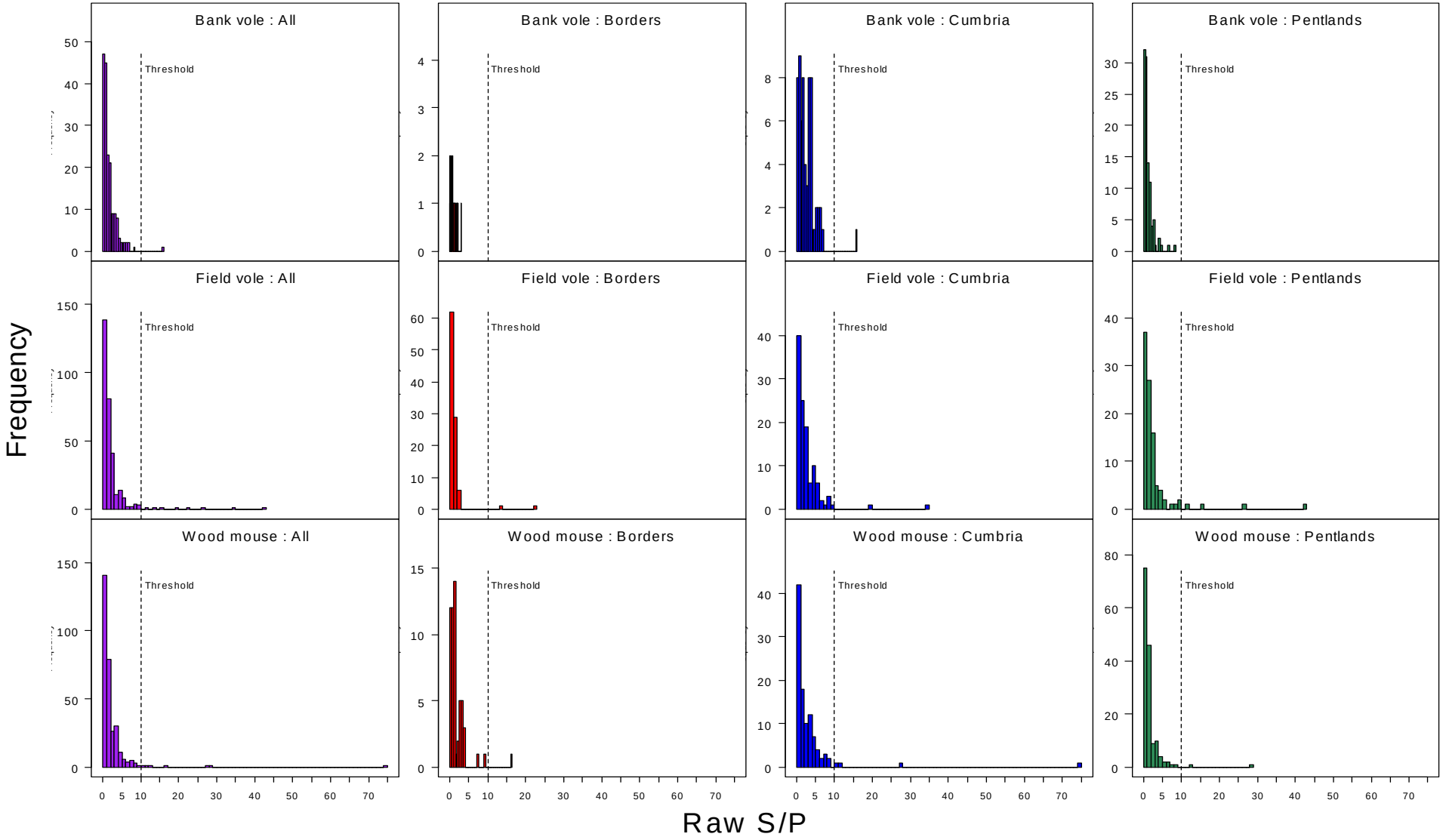
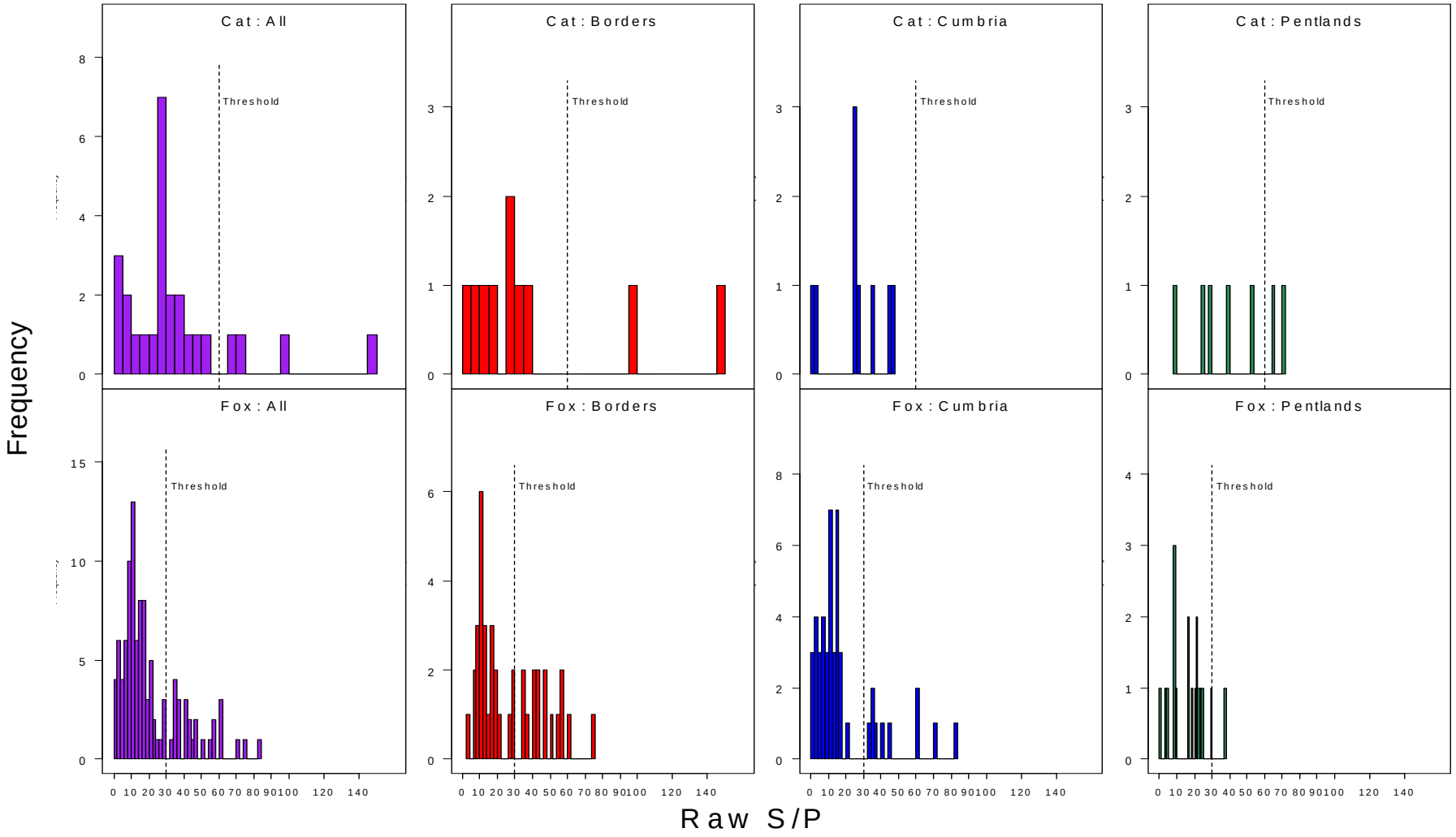


Figure 5.11 Distribution of S/P values for predator species. Dotted line at 60% for cats and 30% for foxes indicates the selected threshold that appears to separate two populations



5.4.3 Seroprevalence

Using the test threshold, as with raw S/P values, seroprevalence was significantly higher in predators (22.7%) than in prey (1.95%) for the study overall ($p<0.001$), and between individual prey (0.54 – 2.56%) and predator species (15.38 – 24.53%)($p<0.022$) irrespective of study area (prey 0 – 4.04%; predators 0 - 37.5%) (Tables 5.5 and 5.6). There were no significant differences in seroprevalence between prey species ($p>0.547$), nor between cats and foxes ($p=0.849$), but in Cumbria cat seroprevalence was 0%.

Table 5.5. Seroprevalence for *Leptospira* spp. (with 95% confidence intervals) for the study overall.

Predator/Prey	Species	N	Seroprevalence
Prey	Bank vole	189	0.54 (0.01 - 2.96)
	Field vole	315	2.56 (1.11 - 4.97)
	Wood mouse	316	2.24 (0.90 - 4.55)
	All	820	**1.95 (1.12 - 3.15)**
Predator	Cat	26	15.38 (4.36 - 34.87)
	Fox	106	24.53 (16.69 - 33.84)
	All	132	**22.73 (15.89 - 30.83)**

Table 5.6 Seroprevalence *for Leptospira* spp. (with 95% confidence intervals) in each species by study area.

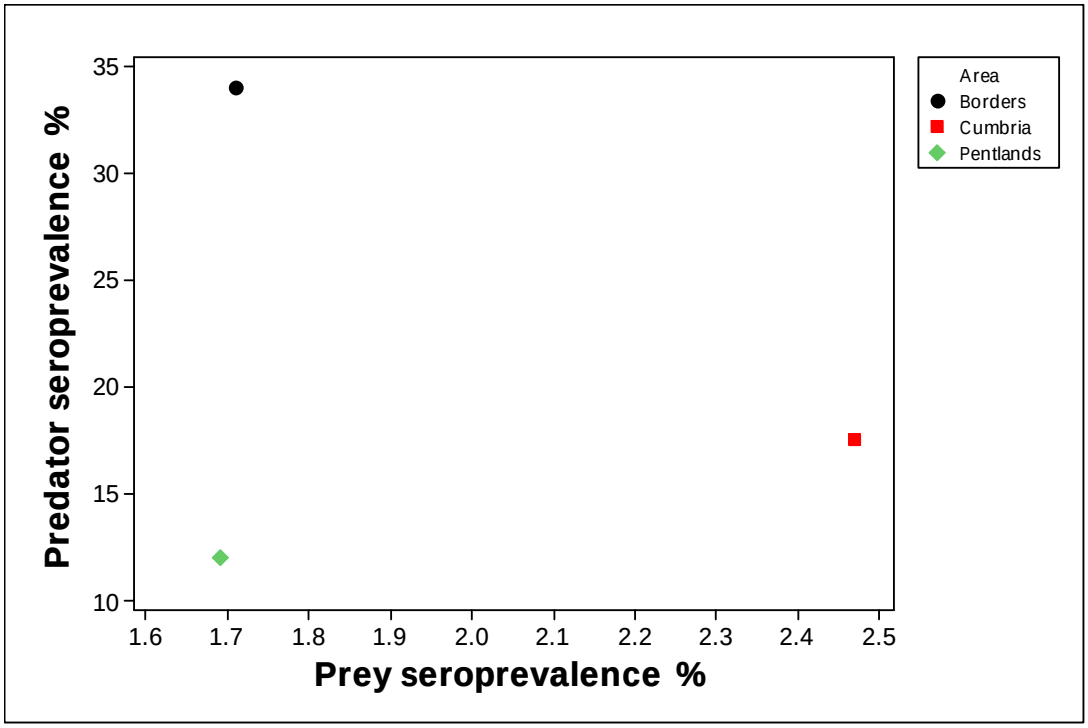
Predator/Prey	Species	Area	N	Seroprevalence
Prey	Overall	Borders	175	**1.71 (0.35 - 4.93)**
		Cumbria	283	**2.47 (1.00 - 5.03)**
		Pentlands	362	**1.69 (0.62 - 3.65)**
Prey	Bank vole	Borders	19	0.00 (0.00 - 17.65)
		Cumbria	64	1.56 (0.04 - 8.40)
		Pentlands	106	0.00 (0.00 - 3.52)
Prey	Field vole	Borders	99	2.02 (0.25 - 7.11)
		Cumbria	115	1.74 (0.21 - 6.14)
		Pentlands	101	4.04 (1.11 - 10.02)
Prey	Wood mouse	Borders	57	1.75 (0.04 - 9.39)
		Cumbria	104	3.85 (1.06 - 9.56)
		Pentlands	155	1.32 (0.16 - 4.67)
Predator	Overall	Borders	50	**34.00 (21.21 - 48.77)**
		Cumbria	57	**17.54 (8.75 - 29.91)**
		Pentlands	25	**12.00 (2.55 - 31.22)**
Predator	Cat	Borders	10	20.00 (2.52 - 55.61)
		Cumbria	9	0.00 (0 - 28.31)
		Pentlands	7	28.57 (3.67 - 70.98)
Predator	Fox	Borders	40	37.50 (22.73 - 54.20)
		Cumbria	48	20.83 (10.47 - 34.99)
		Pentlands	18	5.56 (0.14 - 27.29)

For prey species overall, seroprevalence differed significantly in males (4/412; 0.1%) and females (P=0.016) and was higher in females (12/384; 3.1%). In individual prey species seroprevalence differed by sex in field voles (females higher than males) compared to wood mice but it was not possible to compare with bank voles as no females were seropositive. For predators overall, seroprevalence was not significantly different in males (18/63; 28.6%) and females (5/43; 11.6%)(P=0.0512). It was not possible to compare sex differences in cats as no female cats were seropositive but in foxes seroprevalence did not differ significantly depending on sex (males 31.3%, females 14.7%) (P=0.138)

Only adult prey species were seropositive. In predators, only foxes were seropositive and seroprevalence was not significantly different in adults (12/59; 20.3%) and non-adults (8/25; 32%)(P=0.999), however all the cats tested were adults.

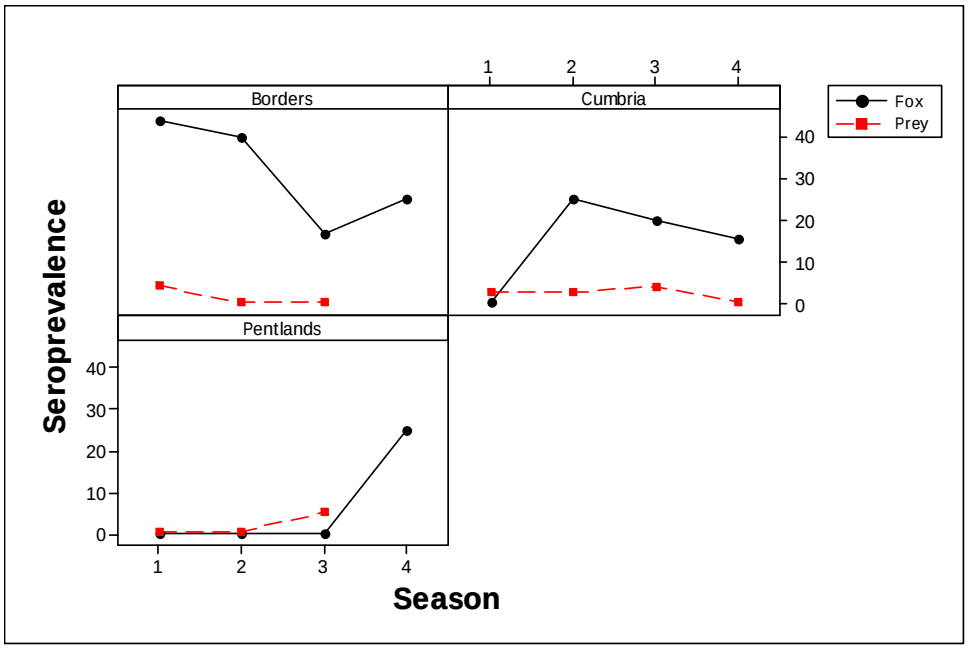
There were no obvious relationships in the pattern of seroprevalence in predator and prey species in the three areas, either when predators were considered alone, or by individual species (e.g. Figure 5.12)

Figure 5.12 Relationship between seroprevalence in predators and prey in the three study areas



Similarly, no consistent patterns were found relating seroprevalence in prey to that in foxes with respect to season (cats were not assigned a season), either for the study overall, or by area (Figure 5.13).

Figure 5.13 Seroprevalence of foxes and prey in the Pentlands area by season, indicating a possible lagged effect between seasons 3 and 4 in Pentlands



In Borders, seroprevalence in foxes declined over seasons 1-3 (44%, 40%, 16.7% respectively) while seroprevalence in prey also dropped from 4.05% in season 1 to 0% in season 2 and remained at 0% in season 3, whereas in Cumbria seroprevalence in foxes increased from 0% in season 1 to 25% in season 2 then declined over seasons 3 (20%) and 4(15%) while seroprevalence in prey oscillated between 0% and 2%. In Pentlands seroprevalence on foxes was 0% through seasons 1-3 then increased to 25% in season 4. Corresponding seroprevalence in prey in Pentlands was 0% in seasons 1 and 2, rising to 1.32% in season 3 (none sampled season 4). These patterns in Borders and Pentlands suggest a possible lagged effect for seroprevalence in the foxes, which rose or fell one season after seroprevalence rose in the prey (Figure 5.13).

Summary of seroprevalence results

In summary, it was found that seroprevalence for *Leptospira spp*. was significantly

higher in predators than in prey, and this pattern was consistent regardless of individual species or area. It also held true when different test thresholds were applied. However, patterns of seroprevalence comparing prey and predators varied by season both overall and in each study area. In the Borders and Pentlands areas there was some evidence for a possible lagged effect in foxes, with their seroprevalence rising or falling one season after a rise or fall in seroprevalence in prey.

5.4.4 Comparison with MAT results

A subset of 232 serum samples from 135 rodents (27 bank voles, 60 field voles, 48 wood mice), 16 cats and 81 foxes were submitted to VLA for testing using the MAT for leptospiral antibodies. When compared to the selected ELISA test thresholds for each species, these comprised 5 rodent samples with an S/P >10% and 130 with S/P <10%, 26 fox samples with an S/P >30% and 55 with S/P<30%, and 4 cats with S/P >60%, and 12 cats with S/P <60%. This subset tested by MAT therefore represented 5/16 (31.3%) of the all the ELISA positive rodents, 26/76 (34.2%) of the ELISA positive foxes and 4/4 (100%) of the ELISA positive cats. A positive MAT result was considered as positive at serovar pool level, whether or not the individual serovars were able to be identified.

Using the MAT test alone, the observed seroprevalence in the subset of samples submitted was 20.0% in prey (95% CI: 13.61 – 27.75) and 38.14% in predators (95% CI 28.47 – 48.57)), which was significantly higher in predators (χ^2_1 =9.30, P=0.002) (Table 5.7).

Table 5.7 Seroprevalence for *Leptospira spp*. using the MAT

Predator/Prey	Species	No. MAT tested	Seroprevalence by MAT (%)
Prey	Bank vole	27	11.11 (2.35 - 29.15)
	Field vole	60	26.67 (16.07 - 39.66)
	Wood mouse	48	16.67 (7.48 - 30.22)
	Overall	135	**20.00 (13.61 - 27.75)**
Predator	Cat	16	12.50 (1.55 - 38.35)
	Fox	81	43.21 (31.82 - 54.09)
	Overall	97	**38.14 (28.47 - 48.57)**

Overall, if the MAT is considered as the gold standard, comparison with the ELISA (at the test threshold of 30% for prey and foxes, 60% for cats) for the subset tested gave a low sensitivity of 29.7% (18.91 – 42.42%) but a high specificity of 90.5% (84.99 – 94.46%) (Tables 5.8 and 5.9).

Table 5.8 Observed frequencies of test results for ELISA compared to MAT

		MAT result		
		Positive	Negative	Total
ELISA result	Positive	19	16	35
	Negative	45	152	197
	Total	64	168	232

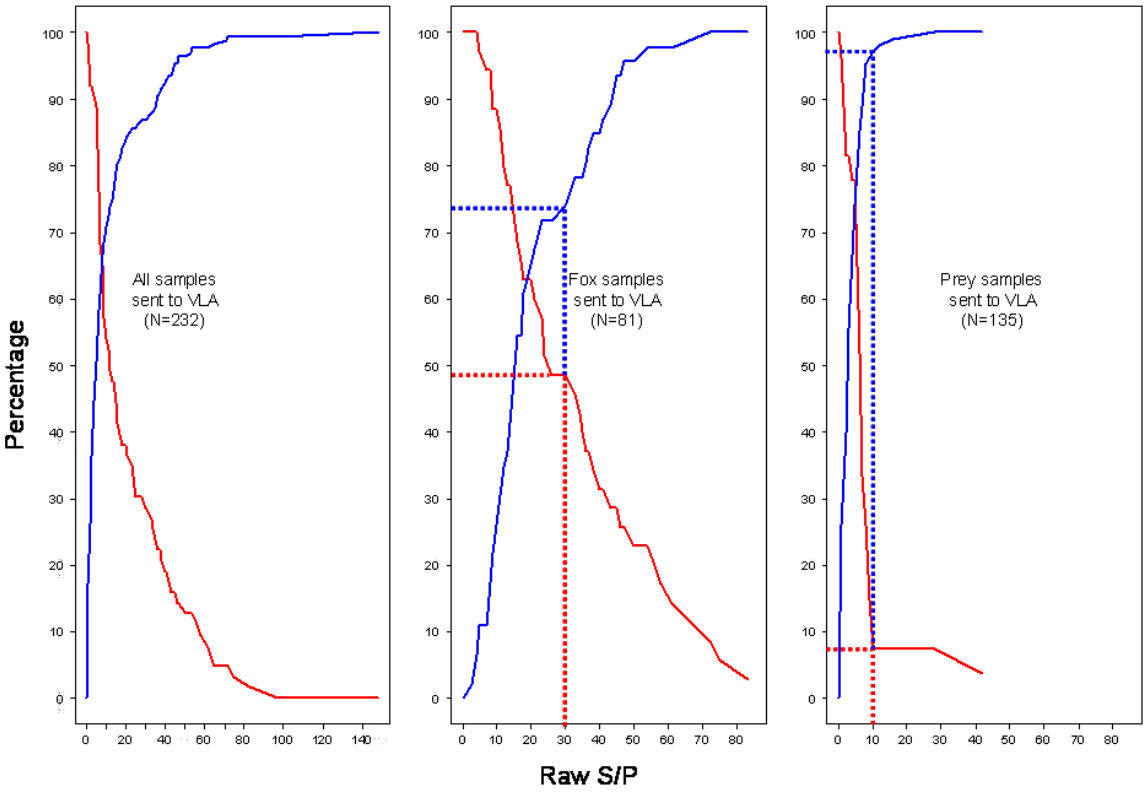
When examined at a species level it was found that, of the 5 ELISA positive rodents, 1 field vole and 1 wood mouse gave true positive results when compared to the MAT, and 25 rodent species gave false negative results (25/130; 19.2%).For the predators, cats had 1/4 (25%) true positives, and 1/12 (8.3%) false negative results when compared to the MAT. However, for the foxes, 16/26 (61.5%) gave a true positive result and 19/55 (34.5%) gave a false negative result. The resultant sensitivities and specificities of the ELISA tests at a species level when compared to the MAT are shown in Table 5.9. Sensitivity is very low in prey, ranging from 0 – 12.5%, but is higher in predators (45.95 – 50%). Specificity is much higher, ranging from 92.5 – 100% in prey and 78.57 – 94% in predators.

Table 5.9 ELISA test sensitivities and specificities (with 95% confidence intervals) for each species when compared to the MAT

Predator/Prey	Species	ELISA sensitivity (%)	ELISA specificity (%)
Prey	Bank vole	0.00 (0.00 - 63.1)	100.00 (88.27 - 100)
	Field vole	6.25 (1.58 - 30.23)	100.00 (93.42-100)
	Wood mouse	12.5 (0.32 - 52.65)	92.50 (79.61 - 98.43)
	All	7.41 (0.91 - 24.29)	97.22 (92.10 - 99.42)
Predator	Cat	50.00 (12.60 - 98.74)	78.57 (49.20 - 95.34)
	Fox	45.71 (28.83 - 63.35)	78.26 (63.64 - 89.05)
	All	45.95 (29.49 - 63.08)	94.0 (83.45 - 98.75)
	Overall	**29.70 (18.91 - 42.42)**	**90.48 (84.99 - 94.46)**

ROC curves plotting sensitivity and specificity of the ELISA in comparison to the MAT describe the overall ability of the ELISA to discriminate positive from negative samples over a range of thresholds (Figure 5.14). These ROC curves illustrate the low sensitivity but high specificity of the ELISA at the test S/P thresholds, especially for prey species (Figure 5.14). Insufficient cat samples were submitted (n=16) to give useful curves. The crossover of sensitivity and specificity curves will maximise both values and is often selected as the test threshold (Dohoo et al., 2003), however, the choice of an appropriate threshold based on the ROC curve will depend on the specific testing or surveillance aims and the outcome or seriousness of obtaining false positive or negative results. In the present study, for the species overall this crossover point is at approximately 10% where sensitivity and specificity are both approximately 65%. Although 10% was used as the threshold for prey when this species group was assessed alone the 10% threshold gave a much lower sensitivity but a very high specificity (Figure 5.14).

Figure 5.14 ROC curves for the ELISA test as compared to the MAT for *Leptospira spp.* (red line = sensitivity, blue line = specificity; dotted lines = species test threshold)



Leptospiral pools and serovars

There were 64 MAT positive tests, and further analysis at the *Leptospira* serovar pool level revealed that 29 animals (2 cats, 13 foxes, 2 bank voles, 12 field voles) were positive for more than one pool. Of these, 26 were positive to 2 pools and 3 were positive to 3 pools. The majority of rodent prey tested positive to pools 4 and/or 6 (Table 5.10). Two cats were MAT positive and both were positive to pools 4 and 6 only. Foxes gave positive results to all pools, with most testing positive to pools 3 and 5. There was no pool for prey (2, 3, 4, 6) that was not represented in the predators, but there were 2 pools in foxes (1 and 5) that were not represented in the prey.

Table 5.10 Number of animals of each species giving a positive result to the *Leptospira* serovar pools 1-6. (29 animals were positive to more than one pool).

Predator/Prey	Species	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6
Prey	Bank vole				2		3
	Field vole		1		14		14
	Wood mouse			6	2		
Predator/Prey	Cat				2		2
	Fox	4	1	19	4	18	3

For a smaller subset of samples (46/64) that gave positive results when tested to the individual serovar level (Table 5.11), there were positive results in both prey and predators to 5 serovars; L. *australis, L. bratislava, L. hebdomanis, L mini and L. sejroe*. 21/46 samples tested positive to more than one serovar. The remaining 18 samples gave either a negative result at the serovar level or there was insufficient sample to test. However, there were three serovars (*L. Pomona, L.hardjo prajitno, L.hardjo bovis*) that were only present in prey, and two (*L.javanica, L. zanoni*) only present in predators.

Table 5.11 MAT results for individual serovars in each species (46 samples)

	Species				
Leptospira serovar	Bank vole	Field vole	Wood mouse	Cat	Fox
L canicola					
L copenhageni					
L ballum					
L icterohaemorrhagiae					
L pomona		1			
L mozdok					
L tarassovi					
L grippotyphosa					
L australis			2		3
L bratislava			3		13
L autumnalis					
L hebdomanis	2	11		1	1
L mini	2	6		1	
L sejroe		1		2	1
L javanica					13
L bataviae					
L zanoni					1
L hardjo prajitno	1	1			
L hardjo bovis	2	5			

Summary of comparison with MAT results

If the MAT is considered as the gold standard, the overall pattern is still that seroprevalence is significantly higher in predators than in prey, which is the same finding as using the ELISA test. In comparison to the MAT the ELISA test has a low sensitivity (28.8%) but high specificity (90.5%) overall but there are marked species differences in test performance. For prey species, test specificity is 97.2% but sensitivity is only 7.41%, whereas in predators, sensitivity is higher (45.95%) and specificity is lower (94.0%). At the serovar pool level, all pools represented in prey were also represented in predators and at the individual serovar level, of the 8 serovars detected, 5 were present in both prey and predators.

5. 5 Discussion

The purpose of this part of the study was to investigate infection with *Leptospira spp*., in order to determine if seroprevalence in predators is higher than in their prey, thereby indicating possible bioconcentration by ingestion or close contact. This

pattern was found, and seroprevalence was significantly higher in predators than prey, irrespective of species and in all study areas. This is a similar finding to that for seroprevalence to *C. burnetii* (Chapter 4), and provides support for the proof of principle of the bioconcentration concept, that predators could act as sentinels for the presence of *Leptospira spp*. in their prey, at least at a pathogen genus level. This finding is backed up by evaluation of the raw data in the absence of a test threshold, which also showed that median S/P values were significantly higher in predator species than in prey species for the study overall and in all three study areas.

In addition, this is also the first report on seroprevalence of *Leptospira spp.* in multiple UK wildlife species (rodents and foxes) and is only the second study to report seroprevalence in cats in the UK. Previous studies of presence or prevalence of leptospirosis in wildlife in the UK are scant. One study from 1969 (Twigg et al., 1969), in which 1,668 mammals of 25 species were examined, reported that 25.8% of wood mice (long-tailed field mice), 19.9% of bank voles and 18.6% of field voles were positive for leptospirosis, as determined by microscopic examination of tissues, but the sample number for each species was not reported. This is much higher than the seroprevalence levels found in the current study, of 1.95% overall for these three rodent species. Little *et al* (1975) report 3/54 (5.6%) field voles as positive using the MAT to the Pomona serogroup and one positive to the Hebdomanis serogroup, and both these serovars were also detected in rodents in the present study. Twigg and Cox (1976) describe the distribution of leptospires in the renal tissues of 69 kidney samples from a variety of UK wildlife species, including rodents and foxes. Hathaway *et al* report infection with serovars of the Australis serogroup as determined by culture and MAT in multiple wildlife species but not as a prevalence (Hathaway et al., 1983a; Hathaway et al., 1983b), and *L. australis* was also detected in both wood mice and foxes in the present study by MAT. Little *et al* (1987) reported serogroup Sejroe, serovar saxkeobing in wood mice, bank voles and field voles, badgers and a fox, and *L sejroe* was also detected in a field vole, 2 cats and a fox in the present study. The most recent study (1995) on wild brown rats by Webster et al reports a seroprevalence of 1% by MAT, 4% by ELISA , 4% by culture and 8% by FAT (14% positive by at least one test) in 259 brown rats from farms in

southern England, with *L. icterohaemorrhagiae* and *L. bratislava* being the only two serovars detected (Webster et al., 1995a). In addition to the serovars previously described above in UK wild rodents, the present study also detected antibodies to *L. mini, L. hardjo* prajitno and *L. hardjo* bovis in UK wild rodents for the first time.

There are no previous published studies on seroprevalence in UK foxes, and this study reports the detection of antibodies to *L bratislava, hebdomanis, javanica* and *zanoni* for the first time in this species in the UK.

In cats, the only UK study reporting seroprevalence that can be identified found that 9.2% of 87 cats sampled in the Glasgow area were seropositive using the MAT (Agunloye and Nash, 1996). This compares to a reported seroprevalence using the MAT of 8.8% in New Zealand cats (Shophet, 1979), and 13.6% in feral cats in Spain (Millan et al., 2009). Antibodies to *L.hardjo* have been decribed in cats previously (Agunloye and Nash, 1996) and were detected in the present study also in one cat (*L. hardjo* bovis), but this is the first report detecting antibodies to *L. mini* in cats.

Although the pattern of a higher seroprevalence in predators than prey was consistent in all three study areas, as found with *C. burnetii* (Chapter 4) there were no consistent patterns in the relationship between seroprevalence in predators and prey that were common to all study areas,. For example it was not found that a higher seroprevalence in prey was reflected by a proportionately higher seroprevalence in predators. This is perhaps not surprising, given that the areas were specifically picked to reflect different habitats, and were of different sizes in terms of area (approximately 176.6 km^2 in the Pentlands, 314 km^2 in the Borders and 380 km^2 in Cumbria) (see Chapter 3, and Chapter 7 for further discussion), and were likely to have many differences in terms of factors such as population density of predators and prey, predation patterns, and predator home ranges.

ELISA development

Commercial ELISA tests are generally aimed at detection of IgM antibodies, by use of an IgM specific conjugate, and, being whole cell based, are able to detect

antibodies against immunodominant carbohydrate LPS epitopes (McBride et al., 2007). The test developed in the present study differs from these in that only extracted leptospiral proteins were used as the antigens, and, by use of a non-species specific protein A and G conjugate, it was aimed at detecting IgG, which is more appropriate for epidemiological studies such as this one, where detection of chronic infection in clinically healthy reservoir hosts is desired. In combination protein A and G should detect most IgG classes and IgM (Akerstrom et al., 1985; Bjorck and Kronvall, 1984; Inganas et al., 1981; Invitrogen, 2011), but is likely to detect mainly IgG as this is the class of antibodies directed against leptospiral proteins. This presumed lack of or poor IgM detection would mean that the ELISA would fail to detect very recent or acute infections, which could lead to an underestimate of seroprevalence, but this was not the aim of the present study. In animals, some similar IgG-detecting ELISAs have been developed, including in cattle (antigens for 1 serovar) (Bercovich et al., 1990),dogs (antigens from 6 serovars) (Ribotta et al., 2000) and wild rodents (antigens for 2 serovars) (Vanasco et al., 2001). In dogs, the IgG ELISA had a sensitivity of 100% relative to dogs with MAT titres > 100 (Ribotta et al., 2000), and in wild rodents sensitivity was also 100% when compared to MAT (Vanasco et al., 2001), indicating that this test approach was appropriate for the present study.

The principles of determination of test cut-offs or thresholds for new serological tests or existing tests used in new species have been discussed in Chapter 4 for *C. burnetii*, and the same process was applied to the results of the Leptospira ELISA developed for this study. As for *C. burnetii*, analysis of the results indicates that different species may have different test thresholds, with cats having a higher threshold. However, the numbers of cats tested were low (n= 26) and greater numbers would be needed for increased confidence in the threshold selected. No references to the use of an ELISA approach to cat serum samples can be found in the literature. Similarly, for foxes, while the number tested was higher (106), greater numbers would improve confidence in the test threshold. However, for all species, it was possible to establish thresholds that could be used to demonstrate a clear difference in seroprevalence between predators and prey.

Comparison of ELISA with MAT results

The MAT is only used as gold standard because of its unsurpassed diagnostic specificity for serovar/serogroup in comparison to other tests (World Health Organisation, 2003), but is recognised as having many drawbacks including being laborious, hazardous (requiring use of live leptospires) and, not least, somewhat subjective, relying on operator assessment of 50% agglutination (O'Keefe, 2002). There are important fundamental differences between the MAT and ELISA meaning that direct comparison and interpretation of comparative results is difficult, as like is not being compared with like.

- MAT relies mainly on LPS serovar determinants on whole live leptospires whereas the ELISA developed in this study is based on extracted leptospiral proteins, which might not be surface-expressed
- Antibodies to LPS are IgM whereas antibodies to proteins are IgG (Guerreiro et al., 2001).
- The MAT can only detect agglutinating antibodies, which can be from both IgG and IgM classes ((World Health Organisation, 2003), whereas the ELISA will detect both non-agglutinating and agglutinating antibodies.

Whether an ELISA is IgM-detecting or IgG -detecting is significant when comparing results to the MAT. In human studies, in comparison to IgM-specific ELISA tests, the MAT has been shown to have relatively low sensitivity, particularly for acute phase antibodies (predominantly IgM),(McBride et al., 2007; Ribeiro et al., 1995; Ribotta et al., 2000). In contrast , in cattle Bercovich et al (1990) reported that IgG was not detected by ELISA until 25 days post infection, compared to 10 days using MAT, and Gerritsen et al (1993) also reported that MAT gave positive results 1-3 weeks earlier post infection than an IgG ELISA (Bercovich et al., 1990; Gerritsen et al., 1993).

Previous studies where both MAT and ELISA have been used have found that seroprevalence is higher when using the ELISA than when using the MAT. For example in Spain seroprevalence in 117 wild rodents of mixed species was found to

be significantly higher by ELISA (47%) than by MAT (40.2%) (Vanasco et al., 2001), and in a study of wild brown rats on UK farms seroprevalence varied between 1% by MAT and 4% by ELISA (Webster et al., 1995a). In contrast, the present study found a seroprevalence of 1.95% by ELISA and 20% by MAT, and in predators 22.73% by ELISA and 38.14% by MAT, however, these results are not directly comparable as the MAT was only performed on a selected subset of samples that were not representative of the total population sampled. The ELISA test used in this study did show low sensitivity compared to the MAT (29.7%) which would suggest it would have limited usefulness as a diagnostic test, and may underestimate true infection levels in a population. ROC curves of sensitivity and specificity against thresholds show that, overall for the species tested, a threshold of approximately 10% gives the combination of maximum sensitivity and specificity, but at this threshold sensitivity is very low in prey and specificity is low in foxes, and it does not correspond to the thresholds for predators as determined by the distribution of S/P values. Increasing the sensitivity of a test means that a higher proportion of false positives will occur, which might be undesirable in certain sentinel or other surveillance programmes, for example if a positive result triggered a particular response. As a screening test in epidemiological surveys such as this one, where presence or absence of enzootic infection in reservoir hosts is required rather than acute infection or reliable diagnosis in clinical situations, the IgG-detecting ELISA is likely to be more appropriate than the MAT, and high specificity (low numbers of false positives) can be useful. Measurement of specificity and sensitivity against a gold standard (MAT) that is recognised to have major limitations, as discussed above, is also questionable, and it would perhaps be preferable to compare the ELISA results with isolation of leptospires from renal tubules by direct or molecular methods, but this was outwith the scope of this study. Nevertheless, the MAT results at Leptospiral pool and serovar levels lend further weight to the proof of principle because all the pools detected in prey were also detected in predators, all serovars detected in prey (with the exception of *L. pomona* and *L. hardjo* prajitno) were also detected in predators, and seroprevalence by MAT was also significantly higher in predators than prey.

In conclusion, this study has shown that for infection with *Leptospira* spp. carnivores can act as sentinels for the presence of this pathogen in their prey and, regardless of the test used (ELISA or MAT), has demonstrated that seroprevalence in predators is significantly higher than in prey. In the next chapter, exposure to the third and final selected pathogen, *Encephalitozoon cuniculi*, is explored, this time using a simple direct agglutination test that gives an immediate positive or negative result, and hence seroprevalence, without the requirement for the setting of test thresholds.

Chapter 6. *Encephalitozoon cuniculi*

6.1 Introduction

The final pathogen selected to test the proof of principle that carnivores can act as sentinels for infectious diseases in their prey was *Encephalitozoon cuniculi*. *E.cuniculi* is an obligate intracellular microsporidian that is the causal agent of encephalitozoonosis, an important and emerging disease in both man and animals, that is known to infect both prey and predator species (Halanova et al., 2003; Mathis et al., 2005; Wasson and Peper, 2000).

Microsporidia are now considered to be highly derived fungi that have one of the smallest eukaryotic genomes described (Katinka et al., 2001). Microsporidia lack mitochondria, but retain enzymes with mitochondrial functions (Katinka et al., 2001), have atypical Golgi apparatus and are considered more closely related to fungi than protozoa, sharing fungal features such as the presence of chitin and trehalose (Muller, 1997). The spores contain a unique long, coiled tubular extrusion apparatus, the polar tube, which distinguishes microsporidia from all other organisms. This polar tube plays a key role in host cell invasion; on extrusion from the spore, it pierces the plasmalemma of a new host cell or the membrane of the phagosome containing the endocytosed spore and injects the sporoplasm and nucleus into the cytoplasm of the new host cell (Franzen, 2005; Xu and Weiss, 2005). The life cycle of *E.cuniculi* is simple and direct, and, like other microsporidia, involves a proliferative merogonic stage, followed by a sporogonic stage resulting in rupture of the host cell and release of small (1.5 x 2.5μm), environmentally resistant, infective spores. Infection is usually horizontal by ingestion or inhalation of spores, which are shed mainly in the urine. Spores are extremely resistant in the environment and can survive for many months in humid environments (Li et al., 2003). Vertical transmission also occurs and is well documented in rabbits, dogs and foxes (Wasson and Peper, 2000).

The main host for *E.cuniculi* is believed to be the rabbit (*Oryctolagus cuniculus*).

E.cuniculi occurs widely in domestic rabbits with various reports of seroprevalence ranging from 15% to 75% in healthy farmed and pet rabbits (Ashmawy et al., 2010; Dipineto et al., 2008; Harcourt-Brown and Holloway, 2003; Igarashi et al., 2008; Okewole, 2008; Santaniello et al., 2009), with one study finding a seroprevalence of 52% in requested samples from healthy domestic rabbits in the UK (Keeble and Shaw, 2006). However, seroprevalence in wild rabbits has been poorly studied. One Australian study found none of 823 wild rabbits to be seropositive to *E.cuniculi* (Cox et al., 1980; Cox and Ross, 1980) , but a later study found 20/81 (24.6%) of wild rabbits seropositive (Thomas et al., 1997). In the UK, only one study in 1979 reports the finding of *E.cuniculi* in wild rabbits, in three out of three rabbits tested from the Pentland hills in Scotland (Wilson, 1979), although a subsequent study in 1980 found no serological evidence of *E.cuniculi* in 175 wild rabbits from other areas of Scotland and England (Cox and Ross, 1980).

In prey species other than rabbits, *E.cuniculi* has been described in laboratory rodents, including in the UK (Gannon, 1980), but prevalence in wild rodents is poorly described. Antibodies to *E.cuniculi* have been detected in 13% (3/23) wild rats in Switzerland (Muller-Doblies et al., 2002), and the organism isolated from the brain of one of these. In an area at the Czech Republic-German Border 14.5% (42/289) faecal samples from wild house mice (*Mus musculus musculus* and *M.m. domesticus*) were positive by PCR for *E.cuniculi* (Sak et al., 2011). Specific antibodies against *E. cuniculi* were found in Iceland in 4% and 9% of *Apodemus sylvaticus* and *Mus musculus* animals, respectively and these rodents were proposed to be a potential reservoir of infection and possible factor in the decline of the arctic fox population in this country (Hersteinsson et al., 1993).

In predators, natural infection with *E. cuniculi* has been reported in domestic dogs in many countries including the UK, South Africa, Tanzania, and the United States (Hollister et al., 1989; Mathis et al., 2005) and three cases have been reported in domestic cats (Canning and Lom, 1986). *E. cuniculi* is a major endemic disease problem in farmed blue foxes in Scandinavia (Akerstedt, 2002), with infection

attributed to ingestion of food contaminated with infected rodent urine or faeces as well as transplacental transmission (Canning and Lom, 1986). However, there is limited information on the disease in wild carnivores in the British Isles, with the only two reports being of *E. cuniculi* detected in the brains of foxes; one wild hand-reared red fox in the UK (Wilson, 1979), and one wild red fox of 33 tested in Ireland (Murphy et al., 2007).

Human infection occurs in immunosuppressed individuals and *E.cuniculi* has emerged as a zoonosis with the advent of HIV/AIDS (Didier et al., 1996; Fournier et al., 2000; Kodjikian et al., 2005; Rossi et al., 1998; Schwartz et al., 1994; Snowden et al., 1999) and has also been described in bone-marrow transplant patients (Orenstein et al., 2005).

In rabbits it has been demonstrated that, although antibodies are produced in immunocompetent individuals in response to infection with *E.cuniculi* and persist for long periods, they do not appear to be protective, and immunodeficient animals do not mount a reliable antibody response (Kunzel and Joachim, 2010). In rabbits a typical response pattern of an early IgM peak followed by prolonged IgG production has been demonstrated after experimental infection (Kunstyr et al., 1986). Antibody detection by serology is the most important tool in the diagnosis of *E.cuniculi* in living animals (Kunzel and Joachim, 2010) and man (Mathis et al., 2005), as histological examination of neural or renal tissue is generally not possible and shedding of spores in urine or faeces, for detection by direct or molecular techniques, can be intermittent.

E. cuniculi was selected as a suitable pathogen for investigation in this study due to its wide host range, known ability to infect both rodents and carnivores, reliance on serological diagnostic techniques and its zoonotic and domestic animal disease potential. In addition, there have been no published reports of *E.cuniculi* in UK wildlife since 1979, and even that was only in three rabbits and one fox, so this lack of knowledge on the current UK status of this pathogen also prompted its

investigation.

6.2 Test options for *E.cuniculi*

Diagnosis of *E.cuniculi* infection is made either by detection of the organism, or the presence of anti- *E.cuniculi* antibodies using serological tests such as ELISA and IFA (6.2.1). Light microscopy combined with special staining techniques (acid-fast trichrome, Ziehl-Neelson) can identify the organism in tissue samples or body fluids, such as urine, to genus level, or electron microscopy techniques can be employed (Mathis et al., 2005). Diagnosis to species level can be achieved using polyclonal or monoclonal antibodies (indirect immunofluorescence microscopy and Western immunoblot assays) (Mo and Drancourt, 2004), or by molecular methods based on PCR, with further analysis of PCR products used to detect strains (Franzen et al., 1998; Katzwinkel-Wladarsch et al., 1997).

6.2.1 ELISA and IFA

The ELISA and IFA are widely used for screening of laboratory animal colonies (Gannon, 1980), farmed and domestic animals (Akerstedt and Kapel, 2003; Goodwin et al., 2006; Santaniello et al., 2009); (Hersteinsson et al., 1993; Hollister et al., 1989) (Ashmawy et al., 2010), wildlife (Cox et al., 1980; Cox and Ross, 1980; Thomas et al., 1997), and for diagnosis of clinical cases in animals and man (Boot et al., 2000; Cray et al., 2009; van et al., 2004). Specific IgM and IgG ELISA are both used in clinical veterinary practice in rabbits to distinguish active, reactivated infection or re-infection from chronic infection (Jeklova et al., 2010).

6.2.2 Direct agglutination test

A direct agglutination test (DAT) methodology has recently been developed (Jordan et al., 2006), which does not require species-specific reagents and avoids the use of any specialised equipment, such as a microplate reader (ELISA) or compound microscope with fluorescent light source (IFA). The DAT was employed in this study due to the requirement in this study to test multiple species, most of which do not have commercially available species-specific antibodies. This methodology has been validated in experimentally infected laboratory mice and has been found to

have high sensitivity (86%) and specificity (98%) for *E.cuniculi* and reduced cross-reactivity to other species of *Encephalitozoon* (*E. intestinalis* and *E.hellem*) when compared to IFA (Jordan et al., 2006). The test has also been used to screen raccoons and beavers (Jordan et al., 2006). In contrast, the IFA has been reported as having significant cross-reactivity with other *Encephalitozoon* species and genetic analysis is often required to confirm the infective species(Jordan et al., 2006). Although an ELISA approach could have been adopted, using a protein A/G conjugate as for *Coxiella burnetii* and *Leptospira* spp. (see Chapters 4 and 5), the positive results from Jordan *et al* (2006) in rodent and carnivore species, high sensitivity and specificity and relative simplicity of the test encouraged the in house creation and use of a similar DAT. Therefore, unlike for the previous two pathogens, no test modification or development of test methodology was required in order to investigate *E.cuniculi*.

6.3 Materials and methods

6.3.1 Direct agglutination test development

E.cuniculi culture and amplification

Madin Darby canine kidney (MDCK) cells were maintained in a biosafety level 2 laboratory. As a precaution, cells were initially treated with ciprofloxacin (10µg/ml) for possible mycoplasmal infection. Cells were cultured and maintained in 150cm^2 flasks at 37°C in a 5% CO_2 incubator, and passaged every 2-3 days using MDCK growth medium (Appendix 6).

Spores of canine subtype species *E. cuniculi* were imported from the USA (American Type Culture Collection, Manassa, VA, USA; ATCC no 50522™). The spores were stored in liquid nitrogen until ready for use, and then thawed by placing the ampoule in a warm water bath at 37°C for approximately 2 minutes without agitation. The thawed solution was removed aseptically and inoculated onto confluent MDCK cells with fresh medium as described above, but without penicillin/streptomycin. Infected cells were maintained in 150cm^2 cell culture flasks in a 5% CO2 incubator at 37°C. The *E.cuniculi* culture was maintained by removing the medium containing the spores every 2-3 days, centrifuging at 1300g for 10 minutes, resuspending the pellet

in fresh medium and transferring this to a fresh flask of confluent MDCK cells. The microsporidia could be visualised with light microscopy within the MDCK cells as refractile intracytoplasmic structures. In parallel to regular passage into fresh flasks, infected flasks were maintained for several weeks, in order to build up large amounts of microsporidial spores. Culture maintenance was carried out over a 6 week period in total (26th October 2009 – 24th November 2009).

In addition to routine passage of spores onto MDCK cell monolayers and regular visual examination with light microscopy, the successful propagation of *E.cuniculi* was also assessed by the establishment of a four-well chamber slide containing MDCK cell monolayers inoculated with supernatant from infected flasks (50 µl per chamber). After 10 days incubation the supernatant was removed and the chamber slide fixed in methanol, and stained with DiffQuik ™, in order to visualise the intracellular organisms and confirm successful infection.

E.cuniculi antigen preparation

E.cuniculi spores were harvested by collecting supernatant from eight 150cm^2 flasks maintained as described above (50ml per flask). The supernatant was centrifuged at 1300g in 25ml aliquots, and the pellets resuspended and fixed in 2ml 4% formaldehyde solution for 10-15 seconds, then diluted with PBS up to 15 ml and stored at 4C overnight to ensure killing of the spores. The fixed spores were then centrifuged at 1300g and the pellets washed in PBS twice to remove the formaldehyde, and the final pellets resuspended in 1ml PBS and pooled to make a total volume of 10ml in PBS. 0.1ml 10% sodium azide was added to this final antigen solution to prevent bacterial growth. Spores were stained with 0.1% SYBR® green 1 (Applied Biosystems, Life Technologies Corporation, California, USA) and counted using fluorescent microscopy.

Validation and testing protocol

The direct agglutination test was conducted in 96-well round-bottom plates. In order to determine the optimum concentration of spores for the test, antigen was prepared from the spore solution (suspension) by making up a solution containing 1 x 10^7 spores/ml using an alkaline-eosin buffer (see Appendix 6) both with and without the

addition of 0.2 mM mercaptoethanol. 0.2mM mercaptoethanol was added to the buffer solution to denature IgM antibodies that may be present in the test serum and prevent non-specific agglutination. Eosin was added to increase visualisation of the agglutination reaction.

A test plate was set up with doubling dilutions of this antigen solution tested in duplicate against a 1:10 dilution in PBS of both a positive control rabbit serum (1:640 titre as obtained by a commercial ELISA, Pinmoore Animal Laboratory Services Ltd) and a negative control normal rabbit serum (R9133, Sigma). 25 µl of control serum was combined with 75 µl antigen solution and mixed thoroughly in each well by pipetting up and down several times. The plate was sealed and incubated at 37°C overnight.

On visualisation over a lightbox, positive sera formed a diffuse opacity across the central portion of the well, whereas negative sera formed a discrete point in the central portion of the well as previously described (Jordan et al., 2006). The optimum concentration for antigen solution was determined empirically as a 1:6500 dilution of the stock solution (10^4 spores/ml). The positive and negative control serum samples mixed with alkaline-eosin buffer that did not contain 0.2M mercaptoethanol all gave a positive reaction, which could be attributed to a non-specific IgM effect and confirmed the need to use mercaptoethanol for the test.

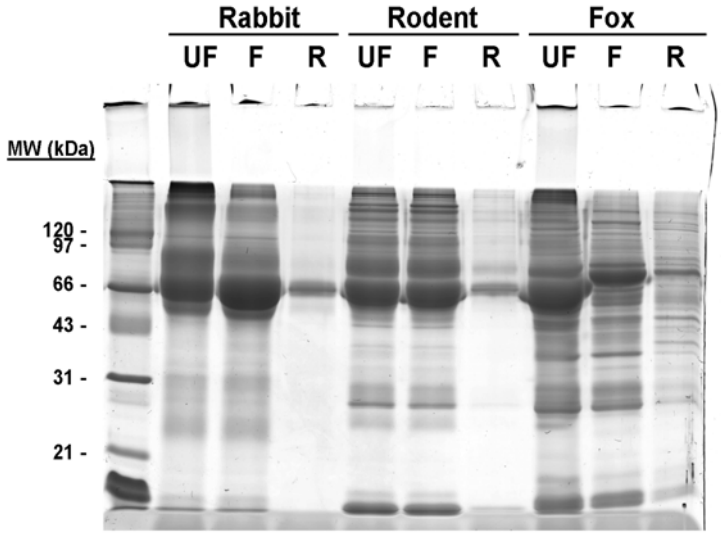
This optimised antigen concentration was then used to determine the optimum dilution for test sera, by titration of the positive and negative control serum. 25 µl of control serum was combined with 75 µl antigen solution and mixed thoroughly in each well by pipetting up and down several times. The plate was wrapped and incubated at 37°C overnight. The positive serum produced easily visible central opacity up to 1:40 dilution, which disappeared at 1:80 dilution. The initial test serum dilution was therefore initially selected as 1:50.

A pilot experiment was performed using sera from 34 rodents and 12 foxes. 5/12 foxes appeared to give a positive result and one rodent gave an equivocal positive

result, but all were hard to interpret in terms of distinguishing a very clear difference between a diffuse opacity and a central point or button at the bottom of the well. There was concern that the poor quality of the fox sera (haemolysed, bacterial and particulate contamination) was influencing the interpretation of results, as poor quality sera itself was opaque and discoloured. Due to this concern the fox sera were then filtered using a 0.2 µm 33mm cellulose acetate sterile syringe filter. The one equivocal positive rodent serum was also haemolysed and so it too was filtered, along with one negative rodent serum, and the test repeated. The filtered fox and rodent serum now gave negative results for all 12 retested samples. In addition, in order to improve the ease of reading the test, it was also decided to increase the test and control serum dilution to 1:25. At this dilution on repetition of the test the positive and negative controls were clearly interpretable. However, filtration of the positive control serum also yielded a negative result by DAT, indicating that filtration was removing some component necessary for the agglutination reaction.

Further investigations of the extracted *E.cuniculi* spore solution using Western blotting with test and positive control serum, and SDS-PAGE of filtered and unfiltered serum, and of material retained on the filter membrane was also performed. These further tests confirmed the significant differences between unfiltered and filtered serum, and retention on the filter of high molecular weight material (e.g. large or aggregated proteins) (Figure 6.1). Therefore the DAT was used with unfiltered serum only, as per the published test protocol by Jordan et al (2006).

Figure 6.1 Reducing gel of DAT positive sera. Rabbit = E.cuniculi positive control serum. UF=unfiltered serum, F=filtered serum, R= recovered proteins from filter.



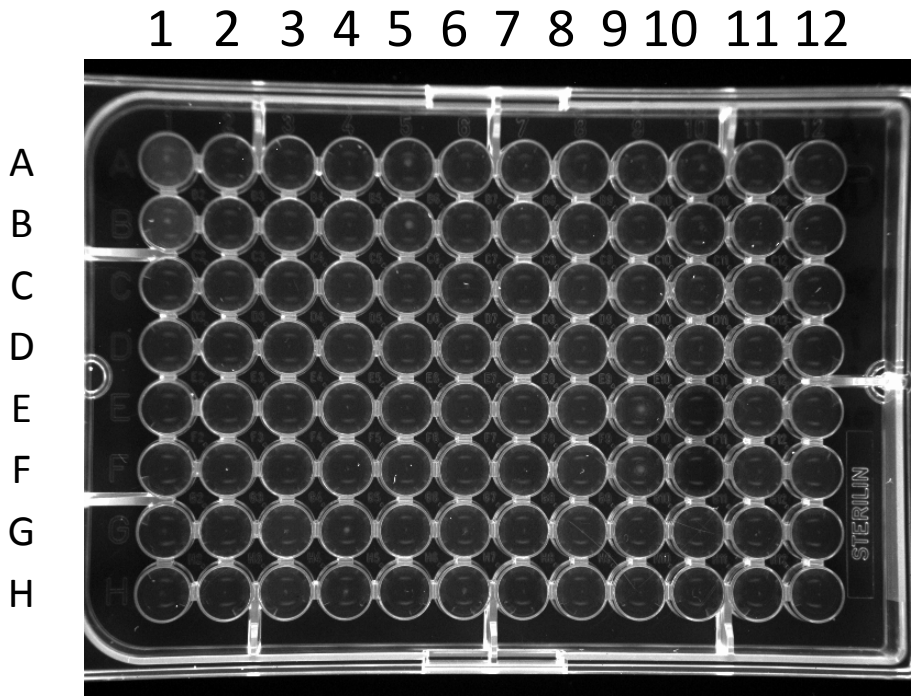
F

Test sera (unfiltered) were diluted 1:25 and 25µl added to 75 µl alkaline-eosin buffer solution and incubated overnight at 37°C. Positive and negative control rabbit sera were tested on each plate. Diffuse precipitate across the bottom of the well was considered a positive agglutination reaction and a central discrete opaque dot was considered a negative reaction, as described by Jordan *et al.* (2006). Photographic images of plates were recorded using a Kodak Image station 440 with UV illumination.

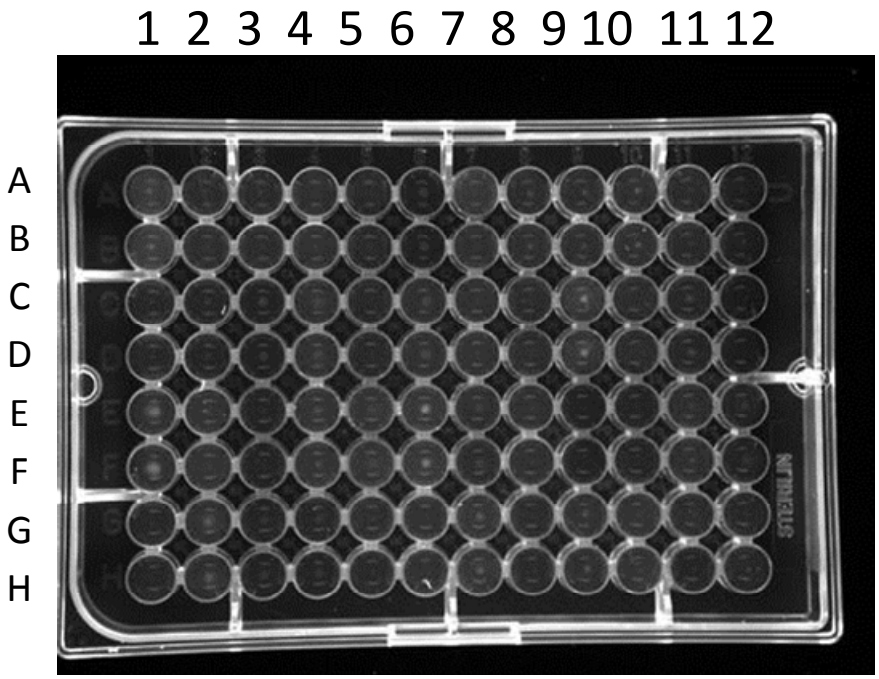
Examples of a direct agglutination test plate using rodent and fox sera are shown in Figure 6.2a and b.

Figure 6.2 Direct agglutination test plate for *E.cuniculi* antibodies in a) rodent serum samples (positive control 1AB, negative control 1CD, positive sera 4GH, 5AB, 9EF and b) fox serum samples (positive control 1AB negative control 1CD, numerous positive sera).

a)



b)



Data analysis

Statistical analysis of the results was performed using R® (R Foundation). Generalised linear mixed effect modelling with binomial errors was used to explore seroprevalence in prey and predator species, with study area as a random effect for all species, and study site nested within study area for prey species. Fixed effects of sex, age, and season were incorporated. For age, animals were classed as either adult or non-adult (juvenile and subadult). Pearson's correlation coefficient (*r)* was used to see if any associations between predator and prey seroprevalence were statistically significant. The significance level was placed at $P<0.05$.

6.4 Results

A total of 921 serum samples were tested for antibodies to *E.cuniculi*, from 793 prey species (178 bank voles, 312 field voles, 303 wood mice) and 128 predator species (27 cats, 101 foxes) (Table 6.1) . This represented 86.96% of the total number of samples of these five species that were collected during the study. The remainder could not be tested due to insufficient quantity of serum. The majority of samples for both prey and predators were collected in seasons 1 and 2, and in season 4 only the Cumbria study area was sampled for prey species.

Table 6.1. Species tested for *E.cuniculi* antibodies in each study area and season

Area	Predator species	Total tested	Season 1	Season 2	Season 3	Season 4	Non seasonal
Borders	Cat	11					11
	Fox	37	23	5	5	4	
Cumbria	Cat	9					9
	Fox	47	1	24	10	12	
Pentlands	Cat	7					7
	Fox	17	10	1	2	4	
	Prey species						
Borders	Bank vole	19	5	14	0	0	
	Field vole	101	47	46	8	0	
	Wood mouse	56	21	3	32	0	
Cumbria	Bank vole	60	11	24	19	6	
	Field vole	109	26	43	19	21	
	Wood mouse	97	40	43	10	4	
Pentlands	Bank vole	99	43	44	12	0	
	Field vole	102	21	55	26	0	
	Wood mouse	150	79	34	37	0	
	TOTAL	**921**	**327**	**336**	**180**	**51**	**27**

6.4.1. Seroprevalence

As the DAT gave either a negative or positive result, these could be translated directly to seroprevalence. Seroprevalence was significantly higher in predators (39.06%) than in prey (5.31%) for the study overall ($p<0.001$) and between individual prey species (1.0 – 10.67%) and foxes (49.5%), ($P<0.018$) (Table 6.2) irrespective of study area (prey 0 – 10.10%; predators 0 – 54.05%) (Table 6.3). Cats as an individual species were excluded from analysis as they were all seronegative. Within individual prey species seroprevalence was significantly higher in bank voles and field voles compared to wood mice ($P < 0.005$).

Table 6.2 Seroprevalence for *E.cuniculi* in predators and prey for the study overall

Predator/ Prey	Species	N	Seroprevalence
Prey	Bank vole	178	10.67 (6.55 - 16.17)
	Field vole	312	5.77 (3.45 - 8.96)
	Wood mouse	303	1.00 (0.21 - 2.89)
	All	793	**5.31 (3.82 - 7.16)**
Predator	Cat	27	0.00 (0 - 10.50)
	Fox	101	49.50 (39.40 - 48.08)
	All	128	**39.06 (30.56 - 48.08)**

Bank voles had the highest seroprevalence of the three rodent species (10.67%), despite the lower numbers caught indicating lower abundance. This compared to 5.77% in field voles and only 1% in wood mice. However, statistically significant differences in prey species seroprevalence were only found between bank voles and field voles compared to wood mice (P<0.001).

Table 6.3 Seroprevalence to *E.cuniculi* in predator and prey species in each study area

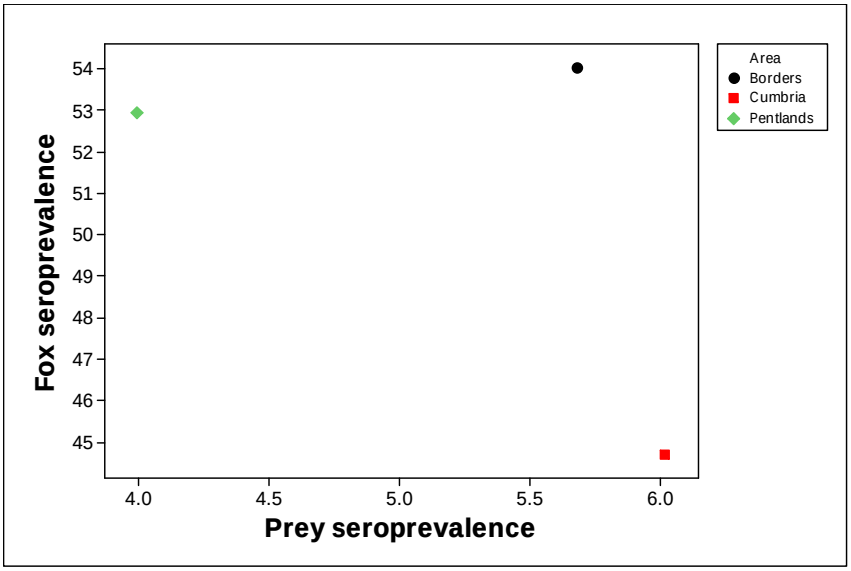
Predator/ Prey	Species	Area	N	Seroprevalence
Prey	Bank vole	Borders	19	15.79 (3.38 - 39.58)
		Cumbria	60	10.00 (3.76 - 20.51)
		Pentlands	99	10.10 (4.95 - 17.79)
Prey	Field vole	Borders	101	6.93 (2.83 - 13.76)
		Cumbria	109	6.42 (2.62 - 12.78)
		Pentlands	102	3.92 (1.08 - 9.74)
Prey	Wood mouse	Borders	56	0.00 (0 - 5.21)
		Cumbria	97	3.09 (0.64 - 8.77)
		Pentlands	150	0.00 (0 - 1.98)
Prey	Overall	Borders	176	**5.68 (2.76 - 10.20)**
		Cumbria	266	**6.02 (3.48 - 9.58)**
		Pentlands	351	**3.99 (2.20 - 6.60)**
Predator	Cat	Borders	11	0 .00 (0 - 23.84)
		Cumbria	9	0.00 (0 - 28.31)
		Pentlands	7	0.00 (0 - 34.81)
	Fox	Borders	37	54.05 (36.92 - 70.51)
		Cumbria	47	44.68 (30.17 - 59.88)
		Pentlands	17	52.94 (27.81 - 77.02)
Predator	Overall	Borders	48	**41.67 (27.61 - 56.79)**
		Cumbria	56	**37.50 (24.92 - 51.45)**
		Pentlands	24	**37.5 0 (18.80 - 59.41)**

For prey species overall, seroprevalence did not differ significantly between males (16/389; 4.1%) and females (22/337; 6.6%) (P>0.12), nor between sexes in individual prey species. For foxes, seroprevalence was not significantly different between sexes (P=0.39). Only adult prey species were seropositive (38/703; 5.4%), and seroprevalence did not differ significantly between adult (32/51; 62.7%) and non-adult (11/17; 61.1%) foxes (P=0.904).

6.4.2 Patterns of seroprevalence

There were no obvious relationships in the pattern of seroprevalence in fox and prey species in the three areas (Figure 6.3), (r = -0.54, P=0.64).

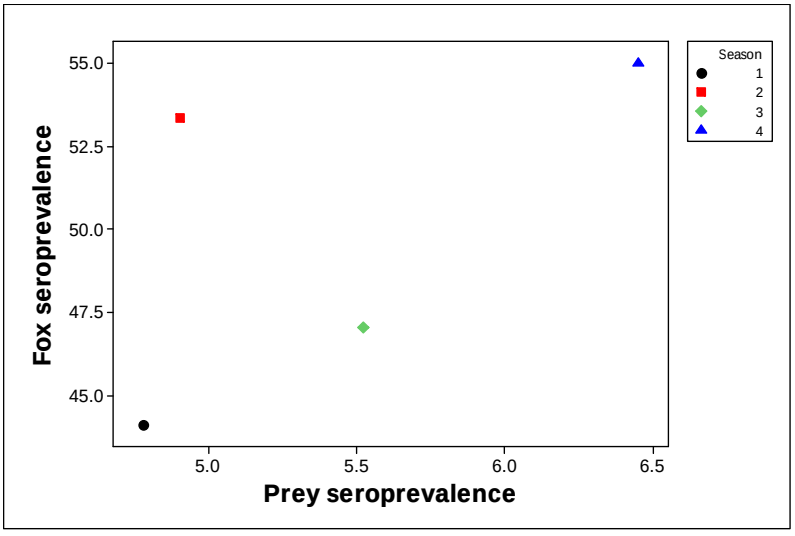
Figure 6.3 Seroprevalence in fox and prey in each study area



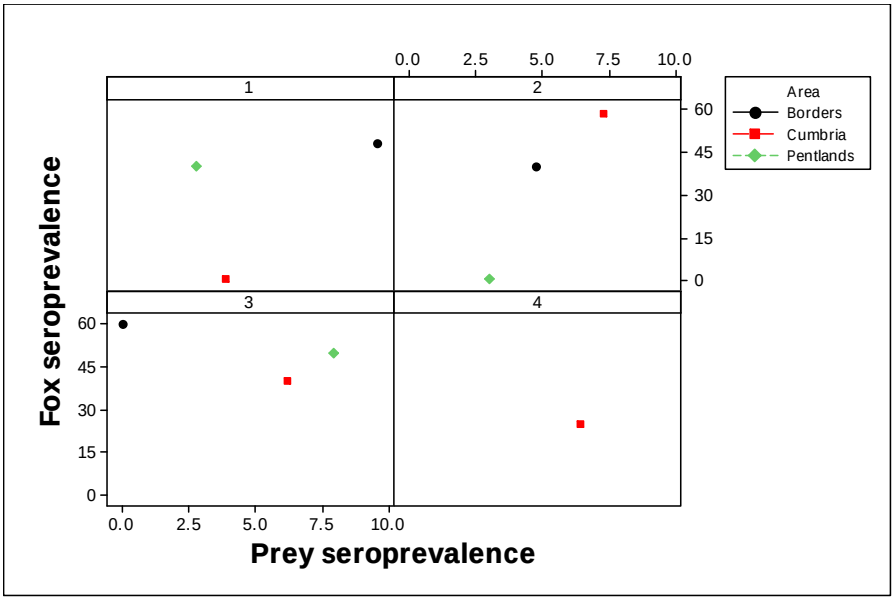
Similarly, no patterns were found relating seroprevalence in prey to that in foxes with respect to season, either for the study overall (r = 0.59, P = 0.42) or by area (r = 0.21, P = 0.56) (Figure 6.4a and b). In Borders, seroprevalence in foxes was always over 40%, whereas in Cumbria and Pentlands there were two seasons where seroprevalence in foxes was 0% (seasons 1 and 2 respectively), however only one animal was sampled in both of these seasons and so the confidence interval is very large (0 – 95%) and this value is unlikely to be representative.

Figure 6.4 Seroprevalence in foxes and prey by season, a) for the study overall, b) by season

a)



b)



6.5 Discussion

This part of the study clearly shows that seroprevalence to *E.cuniculi* is significantly higher in predators (foxes) than in their prey for the study overall and in each study area, thereby providing evidence to support the proof of principle, and indicating possible bioconcentration by ingestion or close contact for this pathogen. This is in agreement with the seroprevalence findings for *Leptospira* spp. described in Chapter 5 and for *C.burnetii* in Chapter 4.

This is also the first report of seroprevalence of *E.cuniculi* in foxes and wild rodents in the UK, indicating that wildlife are a potential reservoir for this infection. *E.cuniculi* has only been detected the brains of one hand-reared fox and three rabbits in the UK, in one study performed over 30 years ago (Wilson, 1979), and it has also been detected more recently in the brain of one fox in rural Ireland (Murphy et al., 2007). Cats were not found to have any evidence of infection with *E.cuniculi* in the present study. There are only three case reports of *E.cuniculi* associated with clinical disease in cats in the literature, in South Africa (van Rensburg and du Plessis, 1971), the USA (Buyukmihci et al., 1977) and Germany (Csokai et al., 2010). In addition, a seroprevalence of 24% (17/72) of cats has been reported in Eastern Slovakia (Halanova et al., 2003) and a recent study in Virginia, USA found a seroprevalence of 6.5% (15/232) (Hsu et al., 2011). *E. cuniculi* is primarily recognised as a disease

of domestic rabbits, so it is unfortunate that it was not possible to test wild rabbits in this study; particularly as it has been demonstrated that the seroprevalence in domestic rabbits is high, with reported levels of 37%-68% (Kunzel and Joachim, 2010), and UK studies demonstrating a seroprevalence of 23% (Harcourt-Brown and Holloway, 2003) and 52% (Keeble and Shaw, 2006) in healthy pet rabbits. Current seroprevalence in their wild counterparts in the UK remains therefore unknown.

The DAT employed in this study was, as described in the report by Jordan *et al* (2007), relatively quick and simple to perform. It had the advantage over the ELISA tests used for the previous two pathogens, of giving an instant positive or negative result across species, meaning that determination of a test threshold was not required. However, the poor quality of the serum from foxes, due to post mortem sampling, did cause some initial difficulties, as the opacity of the serum and particulate material made detection of the agglutination pellet less clear. However, operator experience in looking at test results quickly overcame these initial difficulties. Filtration of the serum, however, seemed to remove components essential to the agglutination reaction, as it made sera that gave a positive result when unfiltered, including the positive control, change from a positive result to a negative result. Therefore it is important that the original test protocol as described by Jordan *et al*, is adhered to and serum is not filtered, as this appears to lead to false negative results.

In contrast to the two previously tested pathogens, (*C.burnetii* and *Leptospira* spp.) there were marked species differences in seroprevalence in prey as well as predators tested - 1% in wood mice, 5.77% in field voles and 10.67% in bank voles. This is in contrast to *C.burnetii* and *Leptospira* spp., where all three rodent species had very similar levels of seroprevalence (<1% for *Leptospira* spp, 12.05 – 12.78% for *C.burnetii)*. Field voles and bank voles were seropositive in all three study areas but wood mice were only seropositive in the Cumbria study area. Of the two predator species tested foxes had a high seroprevalence of 49.5% compared to 0% in cats. Cats have been shown to raise a detectable IgG response to *E.cuniculi* infection (Csokai et al., 2010; Hsu et al., 2011) so this finding indicates lack of exposure to this pathogen (see Chapter 7 for further discussion).

Sex did not have any effect on seroprevalence in prey species. This is in contrast to *C.burnetii* and *Leptospira* spp, where female prey had significantly higher seroprevalence levels than males. However, in predators similar seroprevalence levels in male and female foxes is consistent with the findings for these two other pathogens. Only adult prey were seropositive, which was also found for *Leptospira* spp, and in foxes age had no effect on seroprevalence, which is consistent with the findings for both *C. burnetii* and *Leptospira* spp.

As for the previous two pathogens, no obvious patterns in the relationship between seroprevalence in prey and predators were consistent between the three study areas. As discussed in the previous chapter, the study areas were selected largely because of their differences, so a lack of consistent pattern is not unexpected, and may reflect the differences in study area size, habitat, prey and predator population densities, predator ranges and hunting patterns (see Chapter 7 for further discussion).

Specific experimental studies demonstrating ingestion of infected prey by a predator as a means of establishing *E.cuniculi* infection have not been performed to the authors knowledge, but ingestion of feed contaminated by infected urine, is well recognised as the main route of transmission for *E.cuniculi* spores (Canning and Lom, 1986), and rodent or fox carcases are also traditionally implicated as a source of infection (Akerstedt and Kapel, 2003), so this is a plausible route of infection for the predator species in the present study. Experimental oral infection of adult arctic foxes has been shown to result in an antibody response that is detectable for at least one year after infection (Akerstedt, 2003).Indeed, the absence of rodents in the diet is cited as a possible explanation for the absence of *E.cuniculi* in wild Arctic foxes in Greenland (Akerstedt and Kapel, 2003), whereas in Iceland, where rodents have been shown to be seropositive, seroprevalence in Arctic foxes varied from 2-27% (Hersteinsson et al., 1993). *E.cuniculi* spores are also highly resistant, and predators are also likely to become infected by ingestion of infected urine in the environment. In farmed blue (Arctic) foxes, and other species such as rabbits, vertical transmission *in utero* is believed to be an important route of infection (Wasson and Peper, 2000).

Arctic fox pups infected in utero also produce a strong humoral immune response (Mohn, 1982), and so serological detection in the foxes in this study could also indicate vertical transmission and may be one means of perpetuating infection in a predator species.

In summary, investigation of exposure to *E.cuniculi* has demonstrated that seroprevalence in foxes is significantly higher than in prey, thus providing evidence for the proof of principle that this species of predator has the potential to act as a sentinel for this particular pathogen. However, evidence of infection with *E.cuniculi* was not detected in cats. In addition, the use of the DAT appears to be a valid, simple and practical approach to rapid screening of multiple wildlife species for seroprevalence to *E.cuniculi*.

In the next chapter, the results from the investigations into all three tested pathogens are evaluated further to assess whether the proof of principle has been achieved and to what extent the study techniques employed give a useful indication of presence and prevalence of pathogens in a given area. In addition, a semi-quantitative economic evaluation of the sentinel concept for detection of pathogens will be explored.

Chapter 7. Effectiveness of the study approach in demonstrating the proof of principle and addressing the key research questions

7.1 Introduction

This study set out to establish evidence for the principle that predators can act as sentinels for pathogens present in their prey. Most carnivorous predators ingest multiple prey species, creating many opportunities for transmission of prey-associated pathogens. Predators may therefore be expected to show higher levels of exposure and infection than prey species, effectively acting as 'bioconcentrators' of infection, and may therefore be cost-effective targets or sentinels for surveillance of these pathogens (Cleaveland et al., 2006). This study has shown that the seroprevalence of three out of the four pathogens originally selected for investigation (*C.burnetii*, *Leptospira* spp. and *E.cuniculi*), was significantly higher in predator (fox and cat) species than in prey species (wild rodents) in three separate UK study areas of varying habitat, although *E.cuniculi* was not detected in cats (Table 7.1).

Table 7.1 Seroprevalence for the study overall for *C.burnetii*, *Leptospira* spp. and *E. cuniculi* (confidence intervals not shown)

		Seroprevalence (%)		
Predator/ Prey	**Species**	***C.burnetii***	***Leptospira* spp.**	***E.cuniculi***
Prey	Bank vole	12.78	0.54	10.67
	Field vole	12.62	2.56	5.77
	Wood mouse	12.05	2.24	1.00
	All	**12.44**	**1.95**	**5.31**
Predator	Cat	30.77	15.38	0.00
	Fox	22.55	24.53	49.50
	All	**24.22**	**22.73**	**39.06**

This finding provides good evidence for the principle that predators can act as sentinels for pathogens present in their prey. However, establishing the proof of principle for the fourth selected pathogen, RHDV, was not possible due to

insufficient prey (rabbit) samples being obtained.
In addition, the study provides the first reports in the UK of:

- seroprevalence of *C. burnetii*, *Leptospira* spp. and *E.cuniculi* in multiple wildlife species (field voles, bank voles, wood mice, foxes),
- evidence of infection with *C. burnetii* in wildlife and cats,
- detection of antibodies to *L. mini*, *L. hardjo prajitno* and *L. hardjo bovis* in wild rodents, and to *L. mini* in cats,
- evidence of infection with *E. cuniculi* in wild rodents and foxes.

7.2 Summary of animal sampling, testing, seroprevalence and co-infection

Animal sampling

Prey sampling required targeted live trapping of rodents, which was labour intensive but easily achievable, and donation of dead rabbits from on-going pest control programmes. Contrary to initial expectations, rabbit pest control was hard to access in two out of three study areas, and only low numbers were acquired from the third area, which precluded incorporation of rabbits, and hence investigation of RHDV, into the study.

Fox samples were acquired from on-going fox lethal control programmes and so used an already present resource which could be effectively exploited. In contrast, cat sampling required donation from practicing veterinary surgeons with owner consent. As a result of low participation of veterinary surgeons, cat sample numbers were relatively low. Corvids were also obtained through lethal control programmes in two areas, but serological test methodologies were not directly applicable to avian serum without further modification and so the utility of this species group as sentinels was not assessed in this study.

Sample testing

C. burnetii antibody testing was relatively straightforward using an adapted ELISA test kit for multiple mammal species (Chapter 4). Testing for antibodies to

Leptospira spp was more complex and involved development of an in-house ELISA test for multiple mammal species (Chapter 5). A subset of samples was also tested commercially by MAT for comparative purposes. Testing for antibodies to *E.cuniculi* employed a previously published direct agglutination test (DAT) validated in rodents, but applicable to, and used in, other mammalian wildlife species (Jordan et al., 2006). However, there were some issues with interpretation of the DAT when using poor quality serum samples from dead predators (Chapter 6). For RHDV testing, insufficient acquisition of rabbit samples precluded further investigation, although previous studies from Germany and New Zealand have demonstrated RHDV antibody detection in foxes that consume infected rabbits and supports their use as sentinels of RHDV in a particular area (Frolich et al., 1998; Parkes et al., 2004) .

Seroprevalence

Seropositivity was detected in both predators and prey for all three of the tested pathogens, suggesting that the criteria for selection of candidate pathogen-prey-sentinel combinations, as described in Chapter 2, were appropriate (Table 7.1). Seroprevalence was significantly higher in predators than in prey for all three pathogens in three study areas. For the study overall, C. burnetti seroprevalence in prey species was remarkably similar in all three species (12.05 - 12.78%) and for *Leptospira* spp, the seroprevalence only ranged between 0.54 and 1.95%. *E. cuniculi* seroprevalence ranged more widely between species from 1.00 - 10.67%. However, none of these species differences in prey seroprevalence was statistically significant. In predators, seroprevalence in cats and foxes was not significantly different between species for *C. burnetii* or *Leptospira* spp. but for *E.cuniculi* there was a marked species difference with cats all being negative and foxes having the highest seroprevalence to any pathogen tested (49.5%).

Although seropositivity to all three pathogens was detected in all study areas, there were species and area differences (Table 7.2):

- Wood mice in the Borders area were seronegative for *C. burnetii* and *E.cuniculi*;

- Antibodies to *Leptospira* spp. were not detected by ELISA in bank voles in 2 study areas, nor in cats in Cumbria;
- Cats were seronegative for *E.cuniculi* in all three study areas.

The study did not therefore find cats to be useful sentinels for *E.cuniculi*.

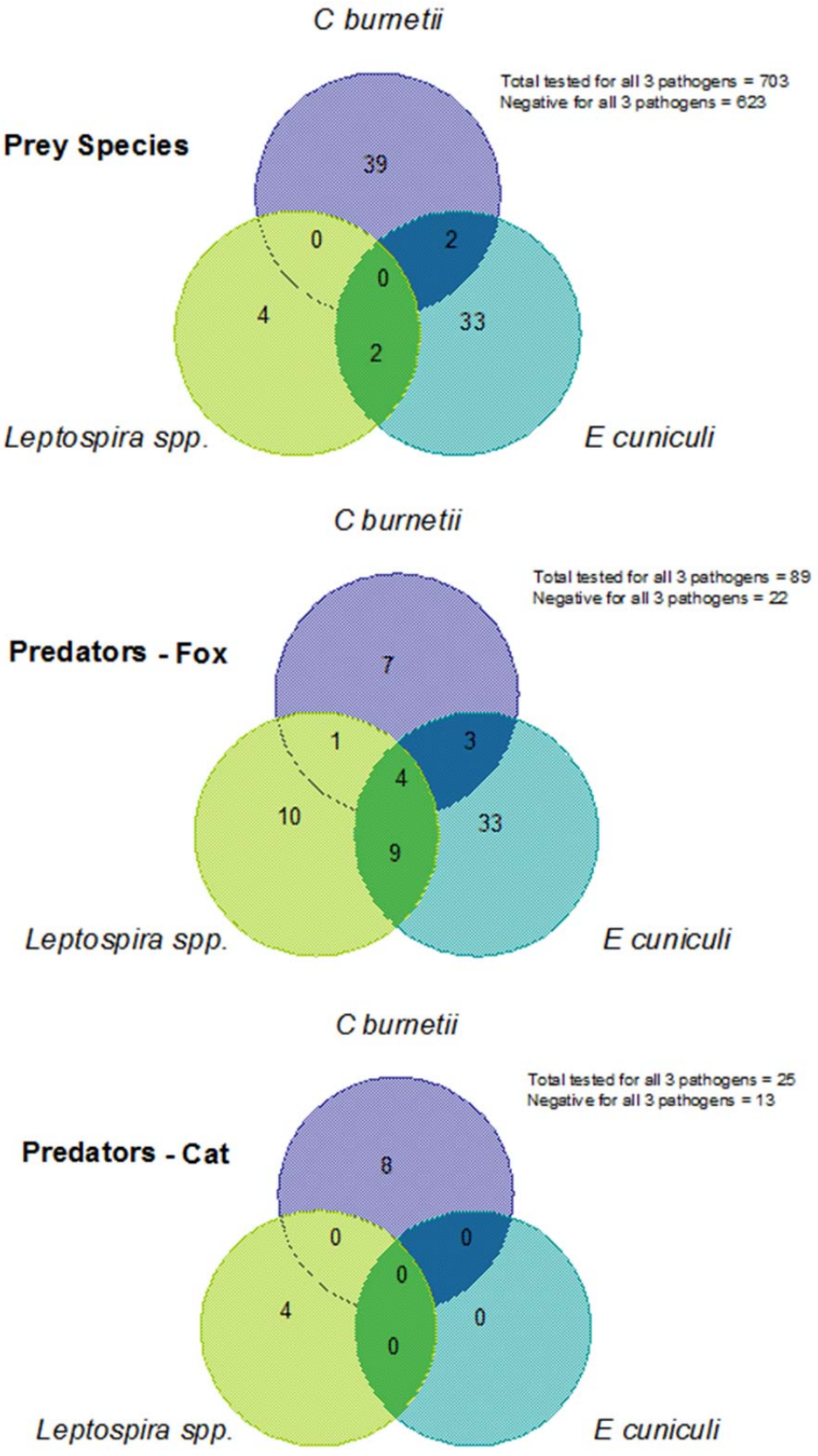
Table 7.2 Seroprevalence in each study area for *C.burnetii*, *Leptospira* spp. and *E. cuniculi* (confidence intervals not shown)

Predator/ Prey	Species	Area	Seroprevalence (%)		
			C.burnetii	***Leptospira* spp.**	***E.cuniculi***
Prey	Overall	Borders	**1.17**	**1.71**	**5.68**
		Cumbria	**9.52**	**2.47**	**6.02**
		Pentlands	**20.17**	**1.69**	**3.99**
Prey	Bank vole	Borders	5.88	0.00	15.79
		Cumbria	8.20	1.56	10.00
		Pentlands	16.67	0.00	10.10
Prey	Field vole	Borders	1.01	2.02	6.93
		Cumbria	9.73	1.74	6.42
		Pentlands	27.84	4.04	3.92
Prey	Wood mouse	Borders	0.00	1.75	0.00
		Cumbria	10.10	3.85	3.09
		Pentlands	17.65	1.32	0.00
Predator	Overall	Borders	**30.00**	**34.00**	**41.67**
		Cumbria	**23.64**	**17.54**	**37.50**
		Pentlands	**13.04**	**12.00**	**37.50**
Predator	Cat	Borders	9.09	20.00	0 .00
		Cumbria	66.67	0.00	0.00
		Pentlands	16.67	28.57	0.00
Predator	Fox	Borders	35.89	5.00	54.05
		Cumbria	15.22	20.83	44.68
		Pentlands	11.76	5.56	52.94

Co-infection

Levels of co-infection were low. Of the 817 animals tested for all three pathogens, only four of the 129 that were seropositive to at least one pathogen (8.2%) were infected with all three (all foxes), and only 17 (four prey and 13 foxes) tested positive for two pathogens (Fig. 7.1).

Figure 7.1 Numbers of co-infections in animals tested for all three pathogens (n=817)



A similar pattern was found for samples that were only tested for two pathogens (n=114), where only 5 (4.4%) (1 prey species, 4 predators) had co-infection. The remaining 191 samples were tested for one pathogen only.

In natural populations, co-infections or sequential infections with more than one parasite species are reported as being common (e.g. (Cox, 2001; Petney and Andrews, 1998; Telfer et al., 2008). Longitudinal studies in field voles have also shown that infection with other parasites has a greater effect on infection risk to other pathogens than other factors such as variations in host susceptibility and exposure (Telfer et al., 2010). In contrast, the present cross-sectional study did not find co-infection with the three selected pathogens to be common in prey species; 95% (76/80) of those tested for all three pathogens and 96% (109/114) of those tested for two pathogens had a single infection. In foxes co-infection was more frequent at 25.4% (17/67), which would be consistent with the theory of infection via consumption of multiple prey species, but in cats co-infection did not occur. Further longitudinal studies and more detailed information on the predation habits and exposure rates of the cats and foxes would be required to elucidate the factors influencing these differences between species and other published studies, but do not form part of the present study.

7.3 Relationships between predator and prey seroprevalence

The focus of this study was to investigate the relationships between seroprevalence in predators and prey to the selected pathogens in order to assess the utility of predators as sentinels. In order to do this, three key questions were addressed, and are discussed below (see 1.2).

- Does sampling predators provide useful information about the presence and prevalence of infection in a given area?
- Does sampling predators provide additional information not available via sampling primary/reservoir hosts alone?
- Is it more cost-effective to sample predators rather than primary/reservoir hosts to detect presence of infection?

Question 1. Does sampling predators provide useful information about the presence and prevalence of infection in a given area?

Yes, the study has shown that where infection was detected in any area it was always found in at least one predator species. Within the three study areas and by individual species the pattern of a significantly higher seroprevalence in predators than prey was consistent. The only exception to this was the finding of higher, but not statistically significantly so, seroprevalence levels for *C. burnetii* in prey compared to predators in the Pentlands area (Table 7.2), where there appeared to be a localised outbreak or pocket of infection in prey species in one of the study sites within this area. If this study site was excluded from analysis the predator and prey seroprevalence pattern in Pentlands was similar to the two other study areas (see Chapter 4).

This overall finding of a higher seroprevalence in predators than prey indicates that, where seroprevalence in prey is very low or undetectable, sampling predators might be a more effective means of detecting the presence of infection (see question 2 below for discussion on sample size). In 8 out of the 9 pathogen/area combinations in this study seroprevalence was statistically higher in predators, and only in one was seroprevalence similar in both species groups, i.e. predator seroprevalence was never found to be significantly lower than that in prey. Therefore, if a similar study was repeated in a different area or for a different pathogen with a similar route of infection (i.e. ingestion), it is not unreasonable to expect that a similar overall pattern between predator (higher seroprevalence) and prey (lower seroprevalence) would be found in that area.

However, although the overall relationship of a higher seroprevalence in predators than prey was similar for all three pathogens, each study area had a unique pattern (Figure 7.3). Possible factors accounting for a lack of consistency across areas include local differences in habitat that influence population size, density and home range of predators and prey (Heydon et al., 2000; Heyman et al., 2009; Lucherini and Lovari, 1996a; Lucherini and Lovari, 1996; Morris and MacEachern, 2010), and the

possibility that ingestion or contact with other prey species in addition to those sampled may be involved in generating the observed predator seroprevalence (see question 2 below).

The usefulness of the information on the presence of infection provided from sampling predators will depend on many factors, including the denominators used for determining seroprevalence, any patterns of seroprevalence found in the different study areas, differences between predator species (e.g. diet and degree of contact with infected prey species), age and sex of animals and sampling methodology.

Denominators

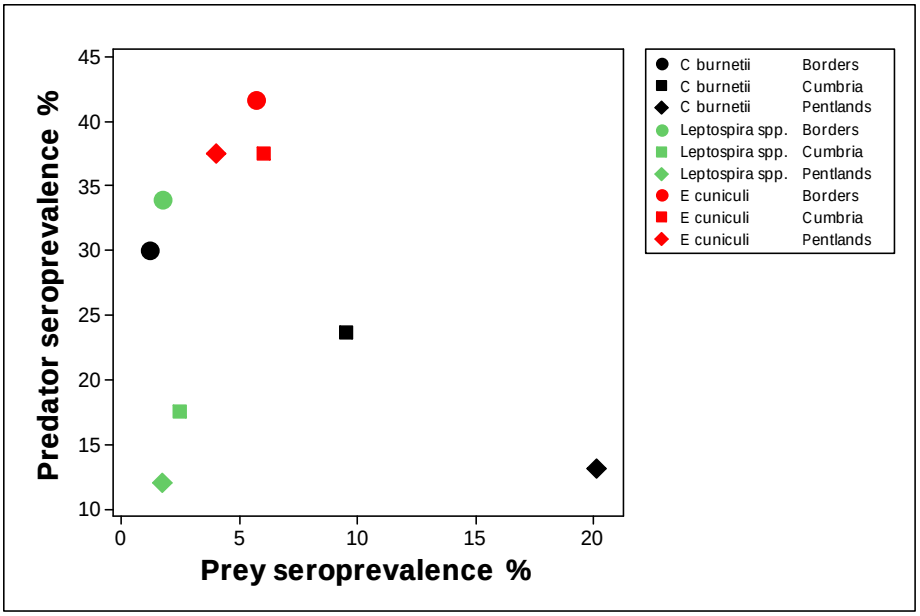
The denominators for seroprevalence in this study were the local populations in each area from which the animals were sampled over the sampling periods, and as these were unknown, the study findings can only be considered an indication of the prevalence in the wider population, as for any study in which a sample rather than the whole population is tested (Dohoo et al., 2003). The representativeness of these samples will affect how generalisable the resultant seroprevalence findings are to the overall population. Many populations of wild animals are essentially continuous across the habitat, and so defining the extent of the local sample population is difficult (Artois et al., 2009). In addition, disease may be aggregated (Artois et al., 2009), such as the apparent pocket of *C. burnetii* detected in rodents in one site in the Pentlands, and in these situations prevalence may therefore be dependent on population density. In addition, other host factors such as age, sex, dominance and reproductive status may be important factors in influencing prevalence of infection (Cross et al., 2009). Seroprevalence is unlikely to be uniform across a continuous population in a particular country or large geographical region, as indicated by the often marked differences found in this study for the same pathogen across the three study areas, (e.g. 1.17%, 9.52%, 20.57% for *C. burnetii* in prey).

Patterns of seroprevalence

Although seroprevalence in predators was consistently and significantly higher than in prey for the three pathogens, this study has shown that it cannot be used to predict corresponding seroprevalence levels in prey in the same area, as no consistent

patterns or positive relationships were found between the two (Figure 7.3) and there was even a significant negative relationship for *C. burnetii* between predator and prey seroprevalence. This negative relationship may be at least partially explained by an apparent localised pocket of high seroprevalence in one particular study site within the Pentlands area (see Chapter 4).

Figure 7.2 Relationships between seroprevalence for all three pathogens in each study area



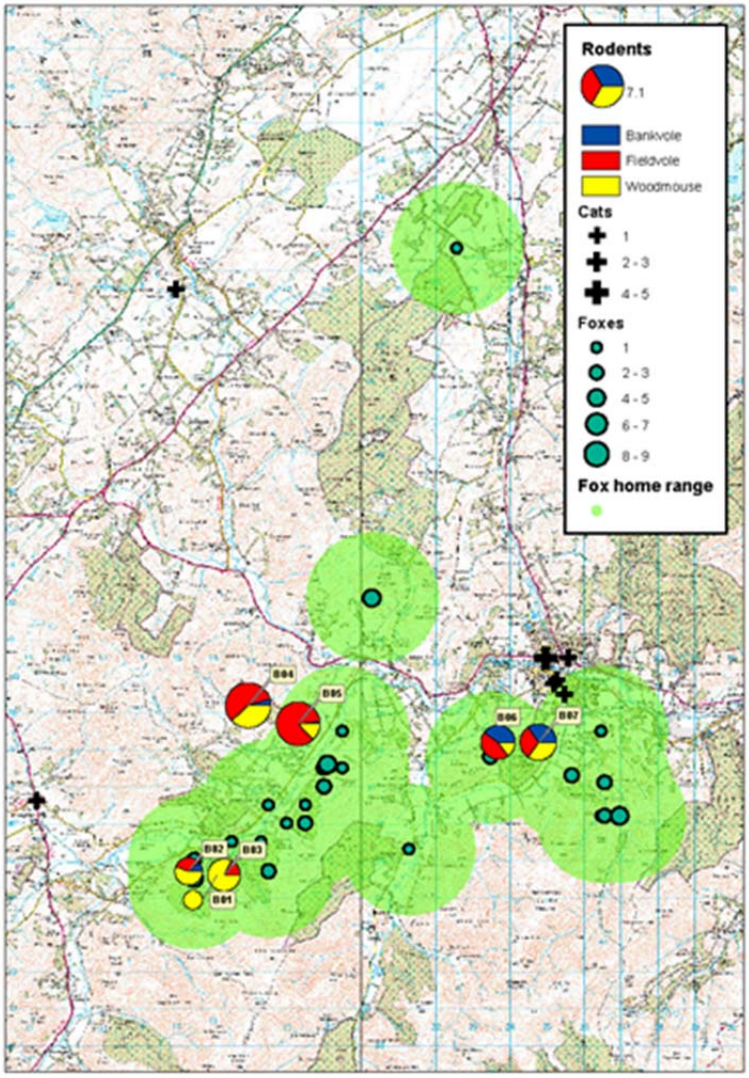
Similarly, no consistent patterns were found for any pathogen relating seroprevalence in prey to that in foxes with respect to season (cats were not assigned a season), either for the study overall, or by area (data not shown). For *Leptospira* spp. there was perhaps some evidence for a possible lagged effect for seroprevalence in the foxes, which rose from 0% to 25% in season 4, one season after seroprevalence rose in the prey from 0% to 1.32%, but this was only seen in one area. However, only crude analysis of these patterns was performed, and other patterns may emerge with more detailed timeline analysis which was beyond the scope of this study, though with only 4 seasons of data there will be limitations to the power associated with such analysis.

Predator species differences

Although predators overall were seropositive to all three pathogens tested in each

area, cats were seronegative to *E.cuniculi* in all areas and seronegative to *Leptospira* spp. in Cumbria. In contrast, foxes were seropositive in all areas. Foxes therefore appear to be more useful in reflecting the presence of these three pathogens in prey species than cats. This may reflect different dietary habits, and different habitats and home ranges that these predators hunt within in the areas selected (e.g. Figure 7.3), and this variation in home range will impact on the degree of overlap and hence potential contact with the wild prey species sampled.

Figure 7.3 (repeated from 3. 17a) Location maps of cats and foxes in relation to trapping grids and fox home ranges (estimated at 10km^2) in the Borders, as an example of the home ranges and different degrees of overlap between prey, foxes and cats



Although domestic cats are known to consume a wide variety of wildlife species (Churcher and Lawton, 1987; Woods et al., 2003), unlike foxes, pet cats do not rely on wildlife as a major source of food, as they are fed by their owners. Cat owners were questioned on the hunting habits of their pet (see Appendix 7.1), and only cats whose owners confirmed that they had directly observed the cat eating prey (including rodents, rabbits and birds) were included in the study. Nevertheless exposure rates may be much lower than that of foxes in terms of the proportion of the diet made up by rodent prey.

One study on cat predation in an English village found an average of 14 prey items per cat per year, of which 17% were wood mice, 7% bank voles and 14% field voles, with garden birds making up 36% of the catch and other mammals including shrews and rabbits 27% (Churcher and Lawton, 1987). Cats are therefore unlikely to be exposed to pathogens present in the prey species tested in this study in the same way or to the same extent as the rural foxes (see below). For example, wood mice are commonly found in urban or semi-urban gardens and even indoors as well as in woodland and other rural environments where cats would have easy access to them, but field voles are found mainly in rough ungrazed grassland or young forestry plantations (Mammal Society 2011), which may be beyond the smaller home range of most pet cats (<1 ha - 27.93 ha), especially if kept in a town (Barratt, 1997; Bradshaw, 1992).

Feral or free-ranging cats were not sampled in this study but are known to have much larger home ranges than their pet counterparts; however, they are still only up to approximately 1km^2 depending on habitat (Fitzgerald and Karl, 1986; Liberg, 1980; Macdonald and Apps, 1978; Turner and Mertens, 1986; Warner, 1985) in contrast to foxes, whose home ranges can be up to 20km^2 in very rural areas (Voigt and Macdonald, 1984). In addition, the relative numbers and accessibility of feral cats for sample collection are likely to be low.

In contrast to cats, foxes are generalist predators and their diet consists of varying proportions, depending on abundance of rodents, lagomorphs, insectivores, birds and

carrion in addition to invertebrates and plant material (Baker et al., 2006; Lanszki et al., 2007; Leckie et al., 1998; Lloyd, 1980; Reynolds and Tapper, 1995). There are differences in the diet between rural and urban foxes, and it has been shown that urban foxes can consume up to half of their diet from anthropogenic sources (Contesse et al., 2004). A recent Polish study concluded that the high percentage of voles consumed by rural foxes (73% of 224 foxes, 47% of food volume) regardless of age, season or habitat, indicated that rural foxes may specialise in preying on these rodents where they are available (Kidawa and Kowalczyk, 2010). However, other studies have found that when rabbits are abundant foxes feed mainly on these (Delibes-Mateos et al., 2008), indicating their opportunistic predation habits. Food intake in foxes is estimated at 388-412g/day (Dyczkowski and Yalden, 1998), which would approximate to 20 rodents weighing 20g each; thus 14 prey items might indeed make up a daily food intake for a fox compared to a year's intake for a pet cat.

Domestic cats were not found to be useful sentinels for *E.cuniculi* in the present study. This could be a feature of the pathogen itself, although recently cats have been shown be able to be infected with *E. cuniculi* and raise a detectable IgG response (Csokai et al., 2010; Hsu et al., 2011). In all three areas, cat seroprevalence to *E. cuniculi* was 0% despite a seroprevalence in prey of 4.0 - 5.7% overall and 10.0 - 15.8% in bank voles (Table 7.2), which could reflect these possible differences in predation patterns, as the seroprevalence was high in all areas in foxes (44.7 - 54.1%). However, some caution is required as the number of cats sampled in this study was small (n = 28, and as a consequence the upper confidence interval for cats was 10.5% (Table 6.2), so this negative finding may not reflect the true situation. *E.cuniculi* seroprevalence was also 0% in wood mice in two areas, so if cats consumed predominately wood mice or other prey rather than the two other species, this could be one possible contributory factor to the lack of *E.cuniculi* exposure in cats. However, in Cumbria cat seroprevalence to *Leptospira* spp was also 0% compared to 20% and 28.6% respectively in Borders and Pentlands, and as the Cumbrian cats came from more rural localities than in Borders and Pentlands (Figure 7.2) the possible explanation of different predation habits is less convincing. In addition, cat seroprevalence to *C.burnetii* was highest in Cumbria (66.7%) where

cats were more rural, compared to the other two areas, and significantly higher than that in Cumbrian foxes (19.6%) (χ^2_1= 10.97, P=0.001).

Without information on the presence of the selected pathogens in the other rodent or wildlife species that domestic cats may consume it is difficult to draw any firm conclusions on how accurately cats reflect pathogen presence in the rodent species tested. Nevertheless foxes, because of their closer association in the habitats where wild rodents were trapped and larger home ranges (Figure 7.2), are probably likely to give a more accurate reflection of pathogen presence in the rodent species trapped and tested in this study than domestic cats. However, if a different population of cats had been sampled that were more closely associated with the sampled prey (such as farm or feral cats), or if prey species more closely associated with the environment of the cats were sampled, or exposure to different pathogens were investigated, a different conclusion might have been drawn.

The above discussion indicates that knowledge of home range sizes of predators is important as it can also allow for reasonable estimates of where infection was acquired. In studies on successful sentinel-based surveillance of bovine tuberculosis in deer in Michigan using coyotes, coyotes were sampled in a known endemic area, and the mean home-range of this species, which is larger than that of the deer, was used as an estimate of where infection was acquired (Atwood et al., 2007). In a similar study in the same area (VerCauteren et al., 2008), it was acknowledged that attempts at spatial correlation of sources of infection for coyotes, sympatric wildlife and domestic livestock would have been confounded because of discrepant home-range sizes and that the presence of an infected coyote could only give a broad indication of the location of the original source of infection. This principle would also be applicable to the foxes and cats sampled in the present study; fox home ranges were estimated at 10km^2 and so infection could have been acquired anywhere within this broad estimated area, but in reality home ranges will vary widely in size and shape and so these estimates only give a broad-scale approximation of where the sampled foxes could have encountered infected prey. Domestic cat home ranges are so much smaller than foxes that they were not visible on the maps (Figure 7.2).

However, this does mean that if cats are seropositive, the area from which infection was likely to be acquired is correspondingly smaller and hence more accurately determined.

Age and sex of prey and predators

Age and sex of both prey and predators are likely to have an influence on their probability of becoming infected with or exposed to a particular pathogen. For prey species, both adult and juvenile animals were obtained but only 2.7% were juvenile, and so this study largely reflects seroprevalence in adult animals (Table 3.6). Similar proportions of males (49.8%) and females (50.2%) (Table 3.6) were obtained, even though males have been reported as being trapped more easily as they encounter more traps in their larger home range (Flowerdew, 1985). Other wild rodent studies have indicated that males are more commonly infected with pathogens, but there is no clear association with age (Hazel et al., 2000; Telfer et al., 2007). In contrast the present study found that females had significantly higher seroprevalence than males to two of the three pathogens tested (*C. burnetii* and *Leptospira* spp), but also found no association with age. However, targeting a particular sex class of rodent would be impractical and wasteful of trapping effort.

Predators in general live longer than their prey, especially domestic cats with respect to the current study, and so they can have more opportunity to encounter a particular pathogen over their lifetime. In the Iberian Lynx (*Lynx pardinus*), seroprevalence to *Toxoplasma gondii*, believed to be largely from consumption of infected rabbits, has been shown to significantly increase with age (Garcia-Bocanegra et al., 2010), and in the Rocky Mountain cougar (*Puma concolor couguar*), age was the most important predictor of risk of exposure to various feline pathogens, including *Yersinia pestis*, which is maintained in rodent populations (Biek et al., 2006). Continuous exposure to, and repeated sampling of, an infected prey population by the predator should mean that if infection is present, predators are likely to remain seropositive, although titres might vary or decline over time.

In this study, the age of predator did not have a significant effect on seroprevalence

levels for any pathogen, but was limited in that sampled cats were all adults, and foxes could only be classified in crude age categories (adults or juveniles) because analysis of dentine layers, which is required for more accurate age estimation (Roulichova and Andera, 2007) was not performed. Seroprevalence in predators was not significantly different in males and females in this study for any pathogen even though, for most predator species, males generally have a larger territory than females which may influence patterns of exposure to prey and pathogens. For example male free-ranging domestic cats are known to have a home range approximately ten times larger than females (Tabor, 1983). Targeting of specific age- or sex- groups of predators for sampling is possible, particularly for domestic cats, but may be potentially wasteful of sampling opportunities, particularly in wildlife. For this study there was no targeting of specific age or sex groups as mainly adult predators were sampled.

Predator sampling bias

The degree of bias in sampling methodology will affect the relevance of prevalence estimates (Petrie and Watson, 2006). Sampling bias will influence how well these estimates reflect the true prevalence in the population and thus, for the purposes of this study, also how the predator seroprevalence relates to that in the prey population. The degree of bias cannot generally be quantitatively estimated (Artois et al., 2009), for example the degree to which the population of shot foxes differs from those that are not shot. In fact this study used non-random (non-probability based) convenience sampling for predators. Foxes are routinely controlled in many rural areas and are thus carcases are accessible for sampling from a pre-existing source, an important factor when considering sampling and surveillance programmes that might utilise carnivores as this will be a cost-effective means of sample acquisition.

However, this introduces an inherent bias, as decisions will be made on which foxes to shoot using criteria such as location (e.g. proximity to livestock) or age, or merely those foxes that are encountered on a particular date allocated for pest control (L. Walton, personal communication). One potential solution to reducing bias is to increase sample size, however this will not necessarily reduce the degree of bias and

the influence it has on results. For example, if only diseased foxes are targeted, shooting more of these foxes will not reduce any bias resulting from diseased foxes being more likely to be shot than healthy foxes. These biases can lead to either over- or under-estimation of disease prevalence. For example, if only foxes exhibiting skin lesions are targeted and shot, this is likely to lead to an overestimation of the prevalence of sarcoptic mange in the fox population. However, if foxes are shot because they are in close proximity to, and predating, game bird rearing areas, they might underestimate the prevalence of disease in foxes more generally, i.e. those that do not predate largely game birds and rely more on other species such as rabbit and rodents as prey. In this scenario, the shot foxes may be more likely to reflect pathogens present in the game birds, than those in rabbits and rodents. As previously outlined, fox diet depends largely on the relative abundance of particular prey items and they will shift to one prey item, e.g. rabbits, when it is abundant (Delibes-Mateos et al., 2008; Ferrari, 1995), so seroprevalence to pathogens acquired by prey ingestion may vary depending on local habitat and relative prey abundance.

Cat sampling in this study also used a very biased population, i.e. those cats presented to veterinary surgeons, requiring blood sampling, and whose owners could confirm that they hunted and gave consent. These pet cats, if only consuming occasional prey items, are likely to underestimate seroprevalence compared to feral, free-ranging or farm cats that sample the wild rodent population regularly but are much less likely to be presented to a veterinary surgeon.

Ideally, stratified random sampling, where populations are subdivided into homogenous and mutually exclusive strata from which random samples are taken, should be used to minimise bias and increase precision of estimates, and is most widely employed for investigating wildlife populations (Petrie and Watson, 2006). However logistic and economic reasons may preclude this approach for predators and convenience sampling may be the most practical and cost-effective approach (see cost-effectiveness below).

However, in some situations bias can be beneficial and indeed desirable. For

example rabies surveillance concentrates on submission of suspect carcases from animals demonstrating abnormal behaviour, which is more effective at detecting the disease than random sampling would be (Artois et al., 2009). Moreover, for the sentinel approach, where detection of disease presence or absence is the focus, positive biases in the sentinel population are usually desired in order to maximise the likelihood of detecting disease if it is present, in a cost/effective manner (McCluskey, 2003) (see Chapter 8). Indeed a distinguishing feature of populations being usefully defined as sentinels from other populations is that they have attributes that enhance detection of disease (Halliday et al., 2007), such as the classic canary in the mine. Ease of access and availability can be considered one of these attributes, even if it inherently leads to sampling bias.

In answer to the first key question therefore, this study has demonstrated that sampling predators does provide useful information on the presence of a selected pathogen in an area (i.e. is it there?). The accuracy of this information will depend on the home ranges and spatial correlation of predators and prey, the degree of positive or negative biases, and non-random sampling methodology. The usefulness of predators appears to be species- and pathogen dependent, and a close link between the selected predator and prey species is likely to be important. However, from the current study it would appear that measuring predator seroprevalence cannot predict what the corresponding prey seroprevalence will be in any given area, and may not reflect more localised pockets of infection within the area. Different areas have different patterns of seroprevalence between predators and prey that are likely to reflect differences in habitat, population size and density, and more accurate seroprevalence estimates would require more detailed information on both predator and prey density. Therefore, this study methodology probably cannot be used to make spatial comparisons in seroprevalence between different areas, but could be used to assess temporal trends in predator seroprevalence if used repeatedly in the same area.

Question 2. Does sampling predators provide additional information not available via sampling primary/reservoir hosts alone?

Yes, the study was able to show that sampling predators could provide additional information that may not have been detected by sampling the selected prey species alone. For the three tested pathogens, in some areas predators were seropositive when certain prey species were seronegative (Table 7.2). For example, wood mice were seronegative to *C. burnetii* and *E. cuniculi* in the Borders, while foxes had high seroprevalence to these pathogens in this area. Again this suggests that predators can detect the presence of a pathogen in an area by sampling, via ingestion or indirect contact, multiple prey of different species. Some of these prey species may have undetectable levels of seroprevalence if targeted alone, or it would require the sampling of very large numbers in order to detect a pathogen with a very low seroprevalence (see sample size below). For the pathogens tested in this study, foxes appear to reflect more closely the presence of pathogens in the prey species tested than cats.

Seroprevalence in the predators may of course also reflect pathogen presence in prey species not sampled in this study, and this may also explain, at least partially, the lack of any consistent relationship between predator and prey seroprevalence. For example, rabbits and wild birds can make up a significant proportion of the diet of both foxes and rural cats (Churcher and Lawton, 1987; Woods et al., 2003). Unfortunately, rabbits were not acquired in sufficient numbers to be included in this study so seroprevalence of the three tested pathogens was not assessed in this species, nor could the seroprevalence of the rabbit specific pathogen RHDV be investigated. Garden birds were also considered at the outset of the study as a suitable prey species but legal and acquisition issues excluded them from the study design (Chapter 2). Other non-rodent sources must also be considered. For example, for *Leptospira* spp. non-rodent sources of *Leptospira* infection for predators could include urine from infected cattle on farmland and other non-rodent reservoirs such as hedgehogs, with which foxes and cats may also have contact. Thus the higher seroprevalence in predators may not directly reflect the seroprevalence in prey

themselves, but rather the general "load" of multiple *Leptospira* spp. in the environment with which the predator has contact.

Sampling predators therefore has the potential advantage of gaining second-hand access not only to multiple prey species, including those in which pathogen presence or exposure is not studied or is not able to be studied, but also to multiple pathogens at the same time. In addition this sampling can gain information on the general presence and prevalence of a pathogen in an area regardless of the prey species host. For example, a serological survey of cats and dogs in rural Canada enabled detection from a single sample per animal of six rodent zoonotic pathogens (Leighton, 2001). Furthermore, the seroprevalence in the predators could also reflect indirect exposure to pathogens of prey hosts via contact with environmental sources such as water and soil. If so, sampling predators could again provide a general reflection of the presence of a pathogen in an area.

In summary, predators have the potential to detect pathogen presence in the target prey, other non-target prey and non-prey sources of a pathogen to reflect the overall presence or prevalence in an area, and to detect infection when not looking in defined target species. This means that they could be used as a single "one-stop" resource in the surveillance of multiple pathogens from multiple sources, as indicated by the higher proportion of co-infections with all three tested pathogens in foxes (8.2%) compared to prey (0%).

However, a further consideration when assessing the potential utility of sampling predators, rather than prey, is that of sample size, discussed below.

Sample size

One of the main concepts behind this study was that, due to the bioconcentration or bioaccumulation effect, required sample size for predators should be lower than that for prey, which is likely to have an impact on the cost-effectiveness and economic evaluation of the sentinel approach (see 7. 3 and the third key question). A relatively simple sample size calculation to detect the presence or confirm absence of disease

uses the formula (Dohoo et al., 2003) (Equation 7.1):

Equation 7.1

$$n = [1-(\alpha)^{1/D}] [N- (D-1)/2]$$

where:

n = the required sample size,

α = (1-confidence level)

N = population size

D = estimated minimum number of diseased animals in the population (N x minimum expected prevalence)

Sampling in order to detect presence or confirm absence of disease is fundamentally different to sampling to estimate prevalence, as was done in the present study and requires an estimate of the expected minimum prevalence at which the disease exists (Dohoo et al., 2003). Confidence levels are usually placed at 95%, so if the required number n are sampled with no positive results, there is 95% confidence that disease prevalence is below the minimum level specified, which could then be accepted as sufficient evidence of disease absence, depending on the infectious agent.

The absolute size of a free-living wild animal population (N), and indeed the domestic cat population, can only be estimated in most situations, for example from previous survey data or trapping success. As discussed earlier, the denominators for seroprevalence in this study were the local prey and predator populations in each study area. Various methods of population size or density estimation exist, but were beyond the scope of the present study and none were employed. These methods include capture-mark-recapture studies, survey data, including using methods that account for probability of detection of wildlife, and estimates of abundance (e.g. using field signs, faeces or genetic methods such as hair analysis), which all have their limitations (Artois et al., 2009). For example, faecal density counts using transects along linear features has been shown to be a cost-effective means of estimating relative fox density, but it remains unclear how this relates to absolute fox density as proportion of faeces associated with linear features and territorial marking

with faeces needs further elucidation (Webbon et al., 2004).

Estimates of vole and wood mouse populations vary and density of wild rodents is known to be habitat dependent (Heyman et al., 2009) and fluctuates, often cyclically (Lambin et al., 2006). The Mammal Society estimate UK field vole numbers at 75,000,000 with a density of up to 100/ha in the spring and bank voles at 23,000,000 with a density of 25/ha in spring, although densities of bank voles can be as low as 1/ha in some areas (Heyman et al., 2009). Wood mice have been found at densities of 150/ha in Spring (Bengtson et al., 1989), whereas other studies have estimate only 7/ha in deciduous woodland (Montgomery, 1989). Using the data from this study collected not only in spring/summer, but also autumn/winter, trap grid areas of 0.25ha (50 x 50 m), yielded a mean rodent number for the study overall of 12.2 rodents/site (= 48/ha, = 4800/km^2). This did not account for "edge effects", i.e. animals outside the grid being attracted to the grid, and animals within but near the edge of the grid whose home range extends beyond it, thereby increasing the actual area of trapping (Tanaka, 1960). Therefore a conservative estimate of **10 rodents/ha** (1000/km^2) across the seasons would seem appropriate for generalised sample size calculations for the purpose of illustrating comparative sample sizes of prey in relation to predators.

For predators, estimates of the UK cat population are largely derived from household questionnaires (estimated at 10.32 million based on a random sample of 2980 households) (Murray et al., 2010) or interviews performed on behalf of the Pet Food Manufacturers Association (estimated at 8 million based on 2022 household interviews; http://www.pfma.org.uk/statistics/index.cfm?id=83&cat_id=60), but this is a whole country estimate assuming a homogeneous population and does not account for local variation in population density. Fox density estimates again vary depending on habitat and, probably, on local culling practices (Heydon et al., 2000); one UK study using spotlight transect surveys found autumn densities of 0.9 - 2.62/ km^2 in three areas used by a fox "hunt" (Heydon and Reynolds, 2000), and another using faecal density counts found that mean density ranged in rural Britain across different habitats from 0.21-2.23 foxes/km^2 (Webbon et al., 2004) and **2/ km^2** was

used for the sample size calculations.

The sample size calculations used below based on Equation 7.1 assume that the population is uniform, there is homogeneous mixing of infected animals within the population, and that samples are random. As discussed earlier, the real-life situation is much more complex, and likely to increase the required sample size, but these calculations can give estimates for comparative purposes of prey and predator sample sizes for different expected seroprevalence levels with various levels of confidence (Table 7.3).

Given these assumptions, a pathogen with an assumed general seroprevalence of 1% in prey and 40% in predators would require a sample size, in a study area of 100 km^2, of 298 prey species, but only 6 predator species to be 95% confident that, if disease is present, it will be detected. If a 99% confidence level is required the prey sample size rises from 106 to 457, while the predator size only rises from 6 to 9 (Table 7.3).

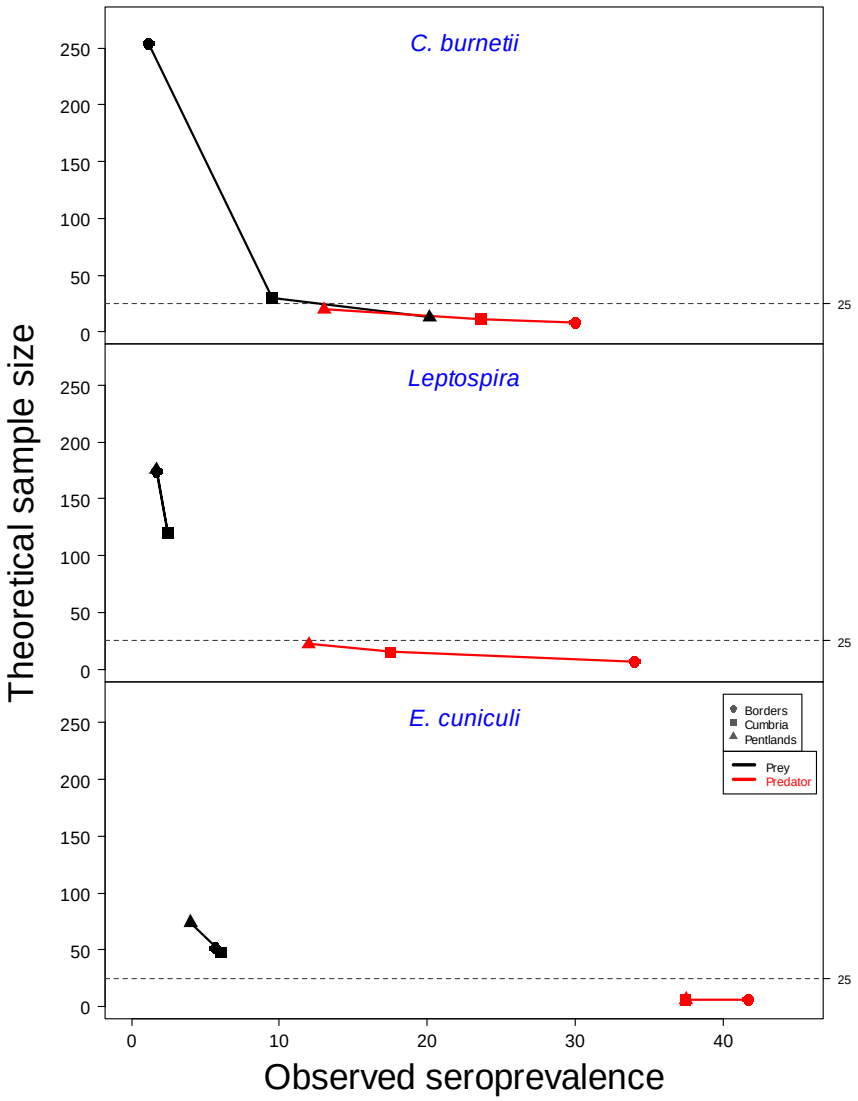
For lower levels of prey seroprevalence, for example 0.01%, the prey sample size is extremely high (26,983) and the likely costs of achieving this level of sampling are likely to be prohibitive (see cost-effectiveness below). If, however, corresponding seroprevalence in predators was 1%, only 155 samples would be required. In the present study 94 foxes were obtained in total so this figure of 155 should be relatively easily attainable, compared to over 26,000 rodents.

Table 7.3 Sample size calculations for absence of disease (95% and 99% confidence) assuming a prey density of 1000/ km^2 , predator density of 2/ km^2 and study area of 100 km2. (* = predator density too low to declare absence of disease at this seroprevalence, as in effect would require sampling of > 2 predators/km^2).

Minimum seroprevalence%	Prey sample size		Predator sample size	
	95% confidence	99% confidence	95% confidence	99% confidence
0.01	26983	36903	*	*
0.1	2969	4499	*	*
0.5	597	915	190	198
0.75	397	610	155	180
1	298	457	155	180
1.5	198	304	126	156
2	148	228	105	136
5	58	90	51	72
10	28	44	27	39
15	18	28	18	26
20	13	21	13	20
25	10	16	10	15
30	8	13	8	13
35	7	11	7	11
40	6	9	6	9
45	5	8	5	8
50	4	7	4	7

Using the actual seroprevalence values found in this study for each pathogen in each area (Table 7.1) and calculating corresponding theoretical samples sizes with the same assumptions on population density and uniform mixing used above, it can be seen that predator sample size is below 25 for all three pathogens (Figure 7.4) whereas prey sample size is higher, up to 250 for *C. burnetii*, with the exception of only 13 prey required for the high seroprevalence of 20.17% for *C. burnetii* found in the Pentlands area.

Figure 7.4 Theoretical sample size of predators and prey required for absence of disease (95% confidence) at observed seroprevalence levels for each pathogen in each study area, assuming a prey density of 1000/km2, predator density of 2/ km^2 and a study area of 100km^2



In reality, as in this study, wildlife sampling is highly unlikely to be random, and is typically clustered (e.g. rodents into grid sites, foxes into the game- keepers patch). This is generally more practical than random sampling as the clusters represent a geographically discrete or compact set of units (Petrie and Watson, 2006). The animals within these clusters will be more similar to each other than if samples were collected randomly from the population (Petrie and Watson, 2006). Clustering will therefore tend to lead to less precise parameter estimates (e.g. seroprevalence) than

those derived from simple random samples of the same number of animals, but more precise estimates can be obtained by sampling a large number of small clusters (e.g. grid sites) rather than a small number of large clusters (Petrie and Watson, 2006). Adjustments for clustering depend on the degree to which test results from within a cluster are similar (the intra-cluster correlation coefficient) and the numbers sampled per cluster (Dohoo et al., 2003). This real-life complexity would need to be incorporated into any sample size calculations for future studies but are beyond the scope of the current study. However, if the effects of clustering are marked and the intra-cluster correlation efficient is high this can lead to a large increase in sample size (Petrie and Watson, 2006).

Therefore, for a surveillance of a particular pathogen, some form of prior knowledge or estimate of the likely seroprevalence in predator species is desirable in determining minimum predator sample size. This was not known or estimated before sampling commenced in the present study for the three tested pathogens, yet seroprevalence was measurable in predators and prey, except for *E.cuniculi* in cats.

However, absence of antibody detection in predators may not of course mean that the pathogen is absent in prey, merely that seroprevalence is likely to be very low (Brinkerhoff et al., 2009), and it may be that if this level is so low it would not be of concern, depending on the surveillance aims and the particular pathogen involved. For example, using the calculated sample sizes presented in Table 7.3, if 180 predators were sampled and all were negative we could be 99% confident that the seroprevalence is less than 1%. Alternatively, absence of detection could mean that insufficient predators were sampled, home ranges may not overlap with those of infected prey (Sangster et al., 2007), ingestion may not be an exposure route that results in seroconversion in the predator, or antibody responses may not persist long enough to be easily detectable for some pathogens, as has been found in one study examining the use of wild carnivores as sentinels for plague (Brinkerhoff et al., 2009). For example, in contrast to the successful sentinel studies for bovine tuberculosis in deer using coyotes in Michigan (Atwood et al., 2007; VerCauteren et al., 2008), a similar sentinel study approach on coyotes in Manitoba did not find *M.*

bovis in coyotes (Sangster et al., 2007) and the authors suggest that this could be attributed to the potential non-overlap of potentially-infected cervids and the trapped coyotes, or the coyote sample size being too low relative to the prevalence rate in cervids.

In the present study an interpretation similar to that of the tuberculosis sentinel study described above was adopted, i.e. that detection of infection was reflective of the study area and the prey population within it in a very general way, despite variation in the degree of overlap between predator and prey home ranges across the study (Figure 7.2) and the relative size of predator and prey sampling areas (Pentlands 176.6 km^2, Borders 314 km^2 , Cumbria 380 km^2). The finding of a similar pattern of seroprevalence in predators (higher) and prey (lower) for all three pathogens across areas in all areas lends support to the present study design as a reasonable and broadly applicable approach to obtaining seroprevalence estimates in an area that reflects, at least, the estimated home ranges of the predators sampled within it. More detailed studies of predator home ranges within a specific area or habitat type would be required to refine this estimated size of area further.

In answer to the second question therefore, this study has shown that carnivores can supply additional information not available by sampling prey alone by being seropositive to pathogens that are at very low or undetectable levels in their prey. In addition, they can detect the presence of multiple pathogens in an area when each prey species may only be positive to one. It is also likely that they can also reflect the presence of a pathogen in an area from other sources including non-target prey species.

7.4 Cost-effectiveness of the study approach

Due to the higher seroprevalence levels compared to prey, the much lower sample size required for predators means that they are likely to be a much more cost-effective and logistically feasible mean of detecting the presence of a pathogen in a given area, especially when it is at low levels of seroprevalence in the prey. This leads to the third key question that this study aims to address:

Question 3. Is it more cost-effective to sample carnivores rather than primary/reservoir hosts to detect presence of infection?

Cost-effectiveness is a key element of sentinel disease surveillance (Centers for Disease Control and Prevention, 2008; McCluskey, 2003). If the costs of sampling predators or testing samples from predators are high relative to prey samples, the benefit of a lower sample size might be outweighed. Therefore when assessing the cost-effectiveness of sampling predators rather than prey, several factors need to be considered:

- Firstly, the costs of acquiring the prey and predator samples, in terms of logistics, time and financial outlay. For any sentinel population to be useful, it must be logistically feasible and safe to sample sufficient numbers of the population (Committee on Animals as Monitors of Environmental Hazards, 1991).
- Secondly, the costs of the selected pathogen test must be assessed. This will largely depend on whether a pre-existing and/or commercially available test is available, whether this can be used immediately or if it requires adaptation or validation for use in non-target species, or whether specific testing methodology has to be developed for the selected species. Time costs are particularly important at this stage, as novel test methodology may take some time to develop and validate.
- Thirdly, once a test methodology is established, the costs of testing the samples, both in terms of man hours and financial expenditure.

There are a number of ways of assessing and evaluating the cost-effectiveness of disease surveillance systems or mechanisms, and no single approach will be applicable to all systems as they will vary depending on the surveillance aim (Drummond et al., 2005). When applied to surveillance systems, cost-based analyses assess value for money by relating the costs and consequence, or outcomes, of two or more surveillance approaches, to inform choices between the various options by decision-makers. For example, for H5N1 avian influenza an evaluation of costs and

effectiveness in terms of the probability of detecting the presence of H5N1 in wild birds, concluded that a sentinel approach of sampling birds found dead was more cost-effective than trapping and testing live birds (Knight-Jones et al., 2010). Types of cost based analyses include: cost minimisation, where equal benefits or outcomes of two or more strategies are assumed; cost-effectiveness, where costs and benefits vary but the benefits can be measured on an equivalent scale; cost-utility analysis, where benefits may not be directly comparable but are translated onto a single scale for comparison, e.g. DALYs (disability adjusted life years) in human medicine which are used to estimate the impacts of disease and benefits of interventions; and cost-benefit analysis, which translates benefits of surveillance into a monetary scale to allow a direct comparison with costs (Drummond et al., 2005).

In looking at the three areas listed above, the current study did not undertake an in-depth economic evaluation or cost-benefit analysis, but adopted a simple and more semi-quantitative approach for the purpose of comparing relative costs between predators and prey. The assumption was that there would be equal benefit from prey and predator sampling in terms of the ability to detect disease presence, i.e. a cost minimisation approach, where the rational choice would be to adopt the cheaper option.

7.4.1 Acquisition and costs of prey samples

Acquisition of rodent samples by trapping required landowner permission for access to appropriate sites, and was successful in all areas, and a total of 74 grid sites yielded 905 rodents over the entire study period. Trapping success (index of abundance) ranged from 6.1 - 36.2/100 trap nights (tn) and this was judged to be acceptable compared to other studies, with reports ranging from 0.2- 10.68/100tn for wild rodent species in different areas and habitats (Augot et al., 2008; Lambin et al., 2000; Leckie et al., 1998; Weihong et al., 1999).

Only three rodent species (field vole, bank vole and wood mouse) were trapped, reflecting the semi-rural or rural nature of the study sites selected and the normal habitat and common occurrence of these species (Mammal Society). If other prey species were to be targeted, such as brown rats (*Rattus norvegicus*) or house mice

(*Mus musculus*) that are more closely associated with human habitation (Mammal Society), this study approach using Ugglan traps placed mainly where vole field signs were seen, would not be appropriate and an alternative trapping strategy focused around farms and farm buildings or domestic habitation would need to be adopted (Parker et al., 2009; Smith et al., 1993; Webster and Macdonald, 1995).

Similarly, less common rodent species, those requiring more specialist trapping methodology or access to very specific habitats, or species that are only active at certain times of year or only found in certain parts of the UK (e.g. dormice (*Muscardinus avellanarius*), harvest mice (*Micromys minutus*)) would require a different approach to sample acquisition. In addition, as these are unlikely to make up any significant proportion of any predator's diet in comparison to the more ubiquitous species trapped in this study, the relevance or applicability of seroprevalence findings may be more difficult to interpret. However, for some particular pathogens of interest, especially if unique to these less common species, it may be desirable to target these species, and the predators that consume them. For example, *Borrelia spielmanii*, an agent of Lyme Disease, seems to be particularly associated with garden and hazel dormice (Richter et al., 2011), and so common predators of this species such as owls, weasels and stoats may be of more relevance than foxes and cats if investigating a sentinel approach for this particular pathogen.

Acquisition of the other selected prey species, rabbits, proved unexpectedly difficult due to the lack of routine rabbit lethal control in the Pentlands and Cumbria areas, and very limited control and acquisition of samples in the Borders area, which was not foreseen at the study design stage and when pathogen- prey-sentinel combinations were selected. This meant it was not possible to investigate seroprevalence in prey for the one selected viral pathogen, RHDV. Investigation into the presence of antibodies to RHDV in foxes and cats was not assessed in the present study but would have been of interest. For such a pathogen, that is host specific (in this case to rabbits only), the ability to obtain the selected host is a vital prerequisite at the planning stage of any similar future studies investigating the proof of principle. However, once the proof or principle is determined for a particular pathogen and

carnivores shown to be appropriate sentinels, sampling of predator species only is required, circumventing this potential problem. For example, studies in New Zealand using released pigs as sentinels for bovine tuberculosis in wildlife indicate that this can be a feasible alternative to direct prevalence surveys for detecting TB presence in possums (Nugent et al., 2002). In the present study, the other pathogens selected (*C. burnetii*, *Leptospira* spp and *E. cuniculi*) are known to have a wide host range and were therefore more suitable for the study design selected here, as it would still have been possible to investigate seroprevalence even if one or more rodent or other prey species were not obtainable.

Trapping efficiency and costs

Although logistically feasible and simple, trapping was labour intensive. In order to assess cost-effectiveness of trapping, a semi-quantitative estimate of trapping efficiency in terms of mean number of rodents trapped per person trapping day was made (Table 7.4). One trapping day was defined as the time taken to check and process the rodents from 6 trapping grids (10 hours).

Table 7.4 Assessment of trapping efficiency in each study area

Area	Person trapping hours	Total rodents caught	Mean rodents/grid site (95% CI)	Mean rodents /person trapping hours
Borders	180	193	10.72 (4.60)	1.1
Cumbria	630	318	8.37 (2.13)	0.5
Pentlands	180	394	21.89 (5.97)	2.2

If the Pentlands figure of 2.2 rodents/person hour is taken as maximum efficiency, the Borders site had a comparative efficiency of 49% and the Cumbria site had a comparative efficiency of just 23%. This was partly due to the use of two people to carry out the trapping in the Cumbria study area. A local assistant was used to pre-bait and set the traps, so that for each trapping session one person was involved for 6 days and one for 3 days. However, even if one person had done all the pre-baiting and trapping in Cumbria, (so 420 person trapping hours overall instead of 630, see Table 7.4) this area would still have been least efficient with a mean rodents/person

trapping hour of 0.8, and efficiency of 35% compared to the Pentlands site. This indicates that there are probably site effects influencing prey sampling, and either local rodent abundance in Cumbria is naturally lower than in the other areas, or choice of habitat and trapping grid location was not optimal. Seasonal trapping efficiency in terms of rodents collected per person trapping hour reduced sequentially per season in all three areas, with the single exception of an increase in Cumbria between seasons 1 and 2 (data not shown). Trapping efficiency declined by 50% between the beginning and end of the study in the Borders, by 27% in Cumbria and by 68% in the Pentlands. This variation between sites and over sequential seasons may need to be taken into account when assessing the cost-effectiveness of other similar studies involving rodent trapping, or for surveillance studies where a known sample size is desired (see trapping costs below).

A semi-quantitative assessment of overall trapping costs was undertaken based on person hours (10 hours/day) (pre-baiting, setting traps, trapping, processing) and estimated costs (trap costs, consumables including bait, sampling equipment, anaesthetic agent) (Table 7.5). Cost per rodent trapped and sampled was assessed to be £12.90.

Table 7.5 Person hours and estimated costs of rodent trapping

Area	Total rodents caught	Person hours	Person hours per rodent	Costs (£)	Costs per rodent (£)	Total cost per rodent at £10/hr
Borders	193	180	0.9	1068.00	5.53	14.53
Cumbria	318	429	1.3	1193.00	3.75	16.75
Pentlands	394	180	0.5	1269.00	3.22	8.22
Overall	**905**	**789**	**0.9**	**3530.00**	**3.90**	**12.90**

These calculations did not include travel time or costs of travel and accommodation incurred during sampling collection, as these would be very much dependent on the location of the sites relative to the research base and would therefore be difficult to generalise to other studies. However, these costs would need to be considered in any detailed economic evaluation of a surveillance programme comparing prey sampling with that of predators. Due to the low trapping efficiency in Borders and use of two

people in Cumbria, costs were approximately twice as high per rodent than in the Pentlands, the area with the highest trapping efficiency (Table 7.4).

7.4.2 Acquisition, quality and costs of predator samples

Predator sample acquisition differed from that of prey because it relied on third parties and could not be as closely planned in terms of dates of acquisition and intensity of effort, as could the rodent trapping. Another problem was obtaining predator samples within close proximity to the rodent trapping sites, particularly for cats, and foxes in the Cumbria study area.

Foxes

For foxes, in the Pentlands and Borders, the personnel shooting foxes were employed by the same farm, local estate or land owner as where the trapping grids were situated, so the overlap of likely home ranges of foxes with the rodent trapping sites was good. However, in Cumbria, this was not the case and a more peripatetic fox control contractor was used. Therefore foxes were obtained from up to 23 km away from the rodent trapping sites, compared to a maximum of 4.2 km in the Borders and 1.7 km in the Pentlands. In the Borders and Pentlands, from local knowledge it was also apparent that compliance was good and the majority of shot foxes were submitted to the study in all trapping areas. In the Cumbria site, it was a compromise between ease of access to land for rodent trapping and the presence of a local assistant, but the finding after trapping had commenced that there was in fact minimal fox control in the immediate area, and so fox samples in Cumbria had to be collected from a much wider area.

However, for future studies where only carnivores would be sampled as sentinels, the issue of having to obtain prey and predators from the same area would not necessarily apply. As long as the location of the predators was known, and ideally estimates of predator density, the required information on pathogen presence could be gained. The main benefit of foxes is that they are already being shot anyway throughout the UK and therefore present an ongoing, easily accessible predator

source that does not require additional input other than arranging a collection system. For serological testing, simple instructions on obtaining a blood sample at the point of killing could be given to the person shooting the fox, and collection, storage and sampling from whole carcases would not be required (see Chapter 8).

Cats

Cat samples proved the most difficult predator samples to obtain and numbers were very low (26 over 2 years). This was largely a result of non-compliance from participating veterinary surgeons, due to non-retention of spare serum for the study when blood-sampling cat patients and/or lack of obtaining informed consent from owners. Repeated reminders were necessary as the study progressed and it was realised insufficient numbers were being donated. Although there were low costs involved with sampling cats (see below) this low sample size meant that confidence intervals for cat seroprevalence were wide (14.3 - 51.8% for *C. burnetii*, 4.36 - 34.87% for *Leptospira* spp., 0 - 10.5% for *E. cuniculi*) and also had an impact on threshold determination of the ELISA tests (Chapter 4 and 5). With hindsight, either a more robust system should have been put in place to ensure compliance, such as weekly or monthly reminders and personal visits to collect samples from the outset. In addition, some sort of incentive scheme could be employed, such as payment per sample or free serum analysis for other parameters relevant to the individual pet animal (see Chapter 8). Alternatively, the Home Office Licence should have been employed and targeted sampling of cats undertaken. However, all these options would have cost, man-power and logistical implications, which could make cats a much less cost-effective option. For future studies an additional option of targeting commercial diagnostic laboratories receiving cat samples from multiple sources and multiple areas of the country could also be employed, and these alternative sources will be discussed further in Chapter 8.

Other predator species

Other mammalian and avian carnivore species (e.g mustelids, raptors, corvids) were not practical choices in this particular proof of principle study for many reasons, mainly a lack of availability through routine culling or legal protection status. In

addition, the development of avian-specific tests e.g. using anti-IgY instead of anti-IgG conjugates, may have both financial and time implications; however, if pre-existing tests exist that are applicable, avian species such as corvids could prove to be cost-effective sentinels for future studies. For example, for *C. burnetii*, a microagglutination test has been used in birds that is also applicable to mammals (To et al., 1998).

Targeted or opportunistic sampling of other carnivore species may be feasible or appropriate in some situations, e.g. trap-neuter-release programmes of feral cats (Levy et al., 2003), targeted mink culling, where mink have already been identified as a potential sentinel for *Toxoplasma gondii* (Sepulveda et al., 2011), or sampling of predators in wildlife rehabilitation centres (Sleeman, 2011), in order to gain information on pathogen presence in the prey of these species in a particular area, or more generally if such programmes are carried out country-wide. For example in 2010 in the US, a plague serosurveillance campaign targeted carnivores across 20 states as indicators of plague activity in rodent populations, and sampled mainly shot coyotes, but also opportunistically sampled and detected antibodies in other carnivores including badger, mountain lion, bobcat, gray fox and black bear (National Wildlife Disease Program, 2010).

Sample quality

Sample quality is an important consideration when obtaining samples from dead animals, if not taken immediately at the point of death or very shortly after. Rapid clotting and haemolysis or bacterial contamination of blood or body fluid could all influence test results and interpretation where quality interferes with a particular test process (Lippi et al., 2008); for example the visualisation of agglutination of live leptospires in the MAT is difficult when the serum contains other live bacteria and particulate matter. Although filtering of serum was used to overcome this for the MAT (by VLA) it was found that filtering altered the response to the DAT when testing for antibodies to *E. cuniculi* (Chapter 6, and see test development below). In terms of antibody persistence in serum from dead animals, it has been shown that antibodies do persist after death, even in decaying carcases up to 11 days post

mortem at ambient temperature (Tryland et al., 2006). Tryland *et al* measured antibodies to *Microsporum canis* in fox serum by ELISA and found that although optical densities were lower as soon as 4 hours post mortem and continued to decline over a period of 11 days after death, they were still measurable. They concluded that positive serological results from testing blood or body fluid of a dead animal is valid, but specific prevalences obtained by screening populations based on samples from dead animals may represent an under-estimation of the true antibody prevalence. In the present study, samples were obtained from dead carnivores within 24 hours and so marked antibody decline was not considered an important factor in determining seroprevalence. However, the effect of antibody decline after death in predator samples, and hence effects on estimation of seroprevalence, should be borne in mind when selecting dead animals as a source of information on pathogen exposure.

Costs

Costs of acquiring predator samples were minimal as they utilised pre-existing sources. A similar semi-quantitative assessment to that used for prey species was adopted. For foxes, carcase collection involved time and mileage costs, but these were minimised by combining collection with trapping trips (Cumbria) or combining collection with other essential journeys (e.g. to and from place of work), and were therefore not assessed as were dependent on location and not generalisable. Time costs were estimated as 0.5 hours to post mortem and process each fox and consumable costs were limited to blood collection (syringe, blood tube) at £0.25/fox. For cats, as samples were excess to those being taken for legitimate veterinary purposes, the only cost was that of the pipette and tube at £0.10/cat. Overall, predator acquisition costs were assessed as £2.68 per predator to obtain and process the sample. For individual species, cats were estimated to cost £0.10 each, and foxes £5.25 each.

Based on these semi-quantitative estimates, the comparative overall costs for this study of obtaining samples were £11,674.50 for sampling prey and £412.72 for sampling predators. Using the derived costs per animal for this study, sample size calculations as in Table 7.3, and the seroprevalence examples previously used above, the estimated cost of sampling for detection of a pathogen with a seroprevalence of

1% in prey and 40% in predators at the 95% confidence level would be £3844.20 for prey (n=298), but only 0.004% of this at £16.08 for predators (n=6). For a pathogen with a seroprevalence of 0.01% in prey and 1% in predators the costs are £348,080 for prey (n=26,983) and just 0.001% of this, £415, for predators (n=155). For these theoretical examples, this represents cost levels of approximately 240 - 840 times greater when using prey compared to predators to achieve the same benefit, i.e. detection of the presence or absence of a pathogen.

Similar indications of the cost-effectiveness of sampling predators were made in the Canadian study on plague by Leighton et al (2001) where previous rodent surveys for plague in the same region, collecting over 20,000 rodents, 60,000 fleas and 2,000 test inoculations over many years to detect a prevalence of 1.8%, are compared with 2.5 months of field work by two people sampling cats and dogs to detect plague seroprevalence at 10%, although estimates of financial cost were not given.

7.4.3 Test methodology and development

The key factor in determining an apparent seroprevalence is the performance of the serological test used, and how accurately this reflects the true seroprevalence in the population under study. A major hurdle with wildlife studies is the lack of validation of diagnostic tests designed for human or livestock pathogens for use in non-target species (Greiner and Gardner, 2000), and the majority have been directly transposed from use in domestic livestock species (Gardner et al., 1996). Only by sampling from animals of known exposure status and/or comparison with a gold standard methodology can the validity of a test be estimated, and for wildlife field studies this is frequently not possible or impractical (Gardner et al., 1996). Even if commercial domestic animal tests are available for the selected pathogens, considerable time may be required for test validation in wildlife species. With the exception of the MAT, new or adapted methodologies were devised for this study (see chapters 4, 5 and 6), with the ultimate aim of providing quick and relatively simple means of testing large numbers of samples from multiple species. The time, resources and expertise required for the test selection and development phase are therefore vital considerations when devising similar studies, or instituting wildlife surveillance

programmes. Obtaining of timely information is also an important component of sentinel surveillance (Centers for Disease Control and Prevention, 2008) and so, for urgent situations when immediate surveillance is required, a cost-benefit analysis of using existing less efficient test methodologies compared to the delays associated with developing new methodology may need to be made.

Each pathogen selected had multiple serological test options, and final test selection was made on the basis of perceived eventual utility in terms of time and cost-effectiveness and the ability to screen multiple samples from multiple species efficiently. If the advantages and disadvantages are considered in light of test pre-expectations and the reality of developing and using the test for wildlife and cat samples once the study had commenced, then Table 7.6 can be constructed to summarise the important test features.

Applicability of the same test to the multiple species under investigation was an important factor for this study, but for future studies using predators as sentinels may not be an issue if only one species is targeted.

For *C. burnetii*, no specific test development was necessary other than discussions with the commercial kit manufacturers to adapt the conjugate from a species specific anti-ruminant conjugate to a protein A/G conjugate (Table 7.6). This is a recognised approach to overcoming the lack of species specific antisera (Worley, 1983) and is a simple method for adapting existing tests or developing new ones for use in wildlife, as was also done for *Leptospira* spp. ELISA methodology has many advantages related to cost effectiveness as multiple samples can be tested rapidly with semi-automation, however the costs of the necessary equipment (e.g. microplate reader) need to be balanced against this. ELISA methodology also has the additional advantage of allowing the use of poor quality serum or body fluids, as the washing stages remove excess serum after binding of antigen and antibody has occurred so it does not interfere with optical density (Table 7.6). Indeed, since the testing of samples in the present study finished, commercial ELISA plates coated with *E. cuniculi* antigen have become available. These potentially could be used with a protein A/G conjugate as an alternative test to the DAT for this pathogen, which would remove the issues of cell culture and antigen extraction, and of reading the

DAT with poor quality serum; it would also have the advantages of semi-automation. However results would need to interpreted in the light of test thresholds rather than producing an immediate positive/negative result as for the DAT and so a cost/benefit assessment of any new methodology such as this would need to be undertaken when considering use in future studies.

Table 7.6 Advantages and disadvantages of the test methodologies used

	C burnetii ELISA	*Leptospira* spp. ELISA	*E.cuniculi* DAT
Advantages			
	Commercial test kit		
	Applicable to multiple species with protein A/G conjugate	Applicable to multispecies with proteinA/G conjugate	Applicable to multiple species
	Semi-automated	Semi-automated	No specialised equipment
	Results require species - specific threshold determination	Results require species - specific threshold determination	Immediate positive/negative result
			Validated in rodent and wildlife species
	Specificity and sensitivity not determined	High specificity compared to MAT, with species differences	High specificity and sensitivity (determined by Jordan et al 2006)
	Serum quality not important	Serum quality not important	
Disadvantages			
		Culture of containment of category 3 pathogen	Importation and cell culture of category 3 pathogen
	Not validated for use in non-ruminant wildlife or cats	Antigen (protein) extraction required	Antigen (protein) extraction required
	Specialised equipment required (microplate reader)	Specialised equipment required (microplate reader)	Poor quality serum can impair interpretation
		Low sensitivity compared to MAT, with species differences	Results may be subjective
		Genus level determination only	

Testing costs

When considering the cost-effectiveness of the test approaches used in this study, only time rather than financial cost was considered. Test development for *Leptospira* spp and *E. cuniculi* involved importation and culture of these organisms under category 3 conditions before antigen extraction could commence (Table 7.6). For *Leptospira* spp. this and the subsequent ELISA development and validation process took approximately 3 months of laboratory time (Chapter 5). Similarly, for *E. cuniculi* time for cell culture, antigen extraction and also investigation into the effects of serum filtration on test methodology was also approximately 3 months laboratory time (Chapter 6).

However, if the development phase is ignored, time taken for testing of samples (not including overnight incubation) was:

C. burnetii: 4 hours/42 samples

Leptospira spp: 6 hours/42 samples

E. cuniculi: 4 hours/42 samples

This was based on the time taken to set-up and process one 96-well plate. Time per sample would depend on how many plates are set-up at the same time; in general for this study 2-4 plates were processed at any one time, so up to 168 samples could be processed in one 4 hour or 6 hour period after a previous overnight incubation. For this study, this means that all the predators obtained (n = 154) could be tested within 2 working days if 4 plates were tested at the same time. However, the testing time for an individual sample is the same for prey and predators alike, although the greater numbers of prey samples required would take longer. For example, the 905 rodents in this study would take 6 times longer to test than the predator samples at these rates, but could still be achieved in 12 days. MAT testing for *Leptospira* spp. by VLA took 30 minutes/8 samples at each stage (screening, pools, serovar) and so time taken per sample would depend on results at each stage (L.Smith personal communication). The above assumes that all samples are collected before testing commences, whereas in many real-life surveillance situations samples would be submitted in varying numbers over time, thus testing may not be as time and cost

efficient if, for example, only a few samples are tested on an ELISA or DAT plate at any one time.

In the present study once the development stage had been achieved, all tests were rapid and enabled multiple samples to be tested efficiently. In a commercial rather than a research setting, further refinements and staff efficiency would be likely to increase the numbers and speed of testing further. A key difference is therefore the time and costs associated with acquiring samples for prey compared to predators, rather than of testing itself, however for diseases of very low seroprevalence in prey, the very large numbers of samples required (e.g nearly 40,000 in the example given above for a seroprevalence of 0.01% at a 99% confidence level) could result in testing times being very prolonged and labour intensive.

In summary, even if a semi-quantitative approach is taken, sampling carnivores/predators is cheaper in a cost minimisation assessment in terms of both time and money than sampling prey, and therefore fulfils a key aim of sentinel surveillance. Hypothetical examples based on the costs incurred in this study show that predator sampling can be approximately 100-400 times cheaper than sampling prey (i.e. 0.1-0.4% of the cost). Careful selection of predator species, methods of sample acquisition and appropriate test methodology are, however, important to maximise this advantage for future studies or surveillance programmes.

7.5 Test performance and validation

A final, but very important consideration of the effectiveness of this study approach, that impacts on all three of the study questions discussed in this chapter, was the performance and validation of the serological tests employed. Test performance can be described in terms of precision (repeatability) and accuracy (e.g. sensitivity and specificity) (See Chapter 2). Due the small amount of serum available from each animal and the necessity to use it for tests for antibodies for multiple pathogens, precision of the ELISA tests (*C. burnetii* and *Leptospira* spp.) was not assessed, other than to test each sample in duplicate in each ELISA test plate. For the *C. burnetii* ELISA each test plate was also assessed by checking that the mean optical

density (OD) of the positive control (ODPC) was > 0.350 and the ratio of the mean OD values of the positive and negative controls was > 3, as per the manufacturer's instructions. The precision of the DAT for *E. cuniculi* was also not assessed other than to include positive and negative control in each sample test plate.

Assessment of test accuracy was also not possible to achieve for two of the three pathogens, as no tests for *C. burnetii* or *E. cuniculi* have been validated on the target species tested, although the DAT has been validated in laboratory rodents and its use published in raccoons, beavers and lemurs (Jordan et al., 2006; Yabsley et al., 2007). However, for most wildlife studies, experimental infection studies in captive populations of each species to investigate antibody and test response are unlikely to be ethical or practical (Gardner et al., 1996). In general, assessment of test sensitivity and specificity is achieved by either i) sampling based on true/known exposure status, ii) sampling based on serological test results followed up by testing of positive and negative individuals by other methods, e.g. culture or molecular techniques or iii) cross sectional sampling with determination of test results and true exposure status in all sampled animals (Gardner et al., 1996). None of these three options were possible within the constraints of the present study and can be difficult to attain for studies of infection in wildlife species in general.

Where it is not possible to assemble sera from animals of known infection status it is possible to estimate diagnostic sensitivity and specificity of tests by "no-gold standard" methods, also known as latent class models, which are complex. Bronsvoort et al. (2008) used a comparison between two serological tests and Bayesian analysis to estimate test sensitivity and specificity and seroprevalence in the absence of a gold standard for foot and mouth disease in wild Cape buffalo, but this type of analysis was outwith the scope of the present study as only one test methodology was available for the two pathogens without an available gold standard, and the approach requires assumptions of prior beliefs in performance of the non-gold standard tests, which are unknown for these wildlife species (Bronsvoort et al., 2008).

Another approach would be to examine tissues from the corresponding animal for

direct evidence of infection in order to aid interpretation of serum antibody levels. Both serological responses and bacterial evidence are required for establishing the presence of infection with *C. burnetii* (OIE, 2008b) and thus this combination could be considered the "gold standard" for Q fever. Astobiza et al (2010) employed a PCR technique on spleen and lung samples to investigate the occurrence of *C. burnetii* in wildlife species in an endemic area in Spain and detected DNA in roe deer, wild boar, hare, vultures and black kites, but not in any wild carnivore species (Astobiza et al., 2010). In addition, they did not assess these animals serologically. Molecular or histological investigation of tissue samples was not within the scope of the present study, but rodent carcases have been retained and future analysis would be warranted. Again, interpretation may be difficult as antibodies could persist after infection has been cleared i.e. the animal could be negative for presence of the pathogen as determined by histological or molecular methods, or culture, but could still be seropositive from previous pathogen exposure. Alternatively, animals could be seronegative despite infection, e.g. if immunocompromised. For example, in rabbits after experimental infection with *E. cuniculi*, persistent IgG levels can be detected in some animals even when the organism cannot be detected in brain tissue by histology, and conversely in others IgG levels can be undetectable despite demonstrable pathogen presence (Kunstyr et al., 1986).

One final approach is exemplified by Drewe et al (2009) who used a combination of two unvalidated serological tests plus bacterial culture to assess disease status of a population of wild meerkats for infection with *Mycobacterium bovis* in the absence of a gold standard for this species. They determined diagnostic accuracy through Bayesian and maximum likelihood estimations of sensitivity, specificity, and likelihood ratios for each diagnostic test when used independently. However, this required a longitudinal study and repeated sampling of the same individuals. In this study it was concluded that, although each test on its own was not diagnostically very useful, when used together they produced estimates of sensitivity and specificity sufficient to inform management decisions (Drewe et al., 2009).

For the one test (*Leptospira* spp. ELISA) where test accuracy could be assessed at

least to some degree by comparison with the MAT, overall sensitivity was found to be low (29.7%), meaning that the ELISA may be underestimating seroprevalence levels if the MAT does reflect "true" levels, although the pattern of higher levels in predators was consistent. However, as discussed in Chapter 5, the results are not directly comparable as only a subset was tested and the use of the MAT as the gold standard can be questioned in the context of use for surveying chronically infected healthy carrier animals, rather than in humans or domestic animals for diagnosing acute infection.

For both ELISA tests used, where the continuous OD data was transformed into a positive/negative result by imposing a cut-off based on frequency distributions using standard methodology, selection of this cut-off is crucial but somewhat subjective, especially where populations of known pathogen status are not sampled (Greiner et al., 1994), and different thresholds may be determined for different species (Ejercito et al., 1993). If the determined threshold was too low, seroprevalence using the ELISA will have been overestimated for this study but only further tests using larger sample sizes or other comparative methods, as described above, could increase confidence in the thresholds used. Although rigorous validation of the tests employed in this study was not possible, the same test was used on both prey and predator species so, if it is assumed that there are no significant differences in serum responses between species, the deficiencies of each test would be applicable to both populations. Therefore the use of these tests, at least as an initial screening process for proof of principle in this study, to compare levels of seroprevalence in predators and prey can be justified in this context.

7.6 Conclusions

In summary, this study has established that predator sampling can detect the presence of a prey pathogen in an area, even when it is not possible to detect it in prey species, and that carnivores can act as useful sentinel species for pathogens maintained in prey species. Seroprevalence levels for the three pathogens tested were consistently higher in predators than prey, but predator seroprevalence could not be used to predict prey seroprevalence. Foxes were assessed to be more useful than cats in

detecting the three pathogens investigated in this study. Due to both lower sample size and costs associated with acquiring samples, predators are a more cost-effective and time efficient option than prey for detection of pathogen presence, but careful selection of species, sampling methods and test methodology are important to utilise carnivores effectively as sentinels.

In the final chapter, all the above is brought together in terms of how predators could be incorporated into future surveillance studies or programmes.

Chapter 8. How could predator sampling be incorporated into future surveillance programmes for endemic pathogens or those representing higher level threats?

8.1 Introduction

The preceding chapters have presented, and discussed in detail, the evidence for the proof of principle that predators can act as sentinels for pathogens present in their prey. Although animal sentinels have appeared to be underutilised generally in infectious disease surveillance (Rabinowitz et al., 2005), in recent years their recognition and use for both human and livestock pathogens has been gathering momentum, particularly where health risks are shared, and the concept of a "One Health" or " One Medicine" approach is increasingly invoked (Schwabe, 1984; (Arambulo, 2011; Kaplan, 2011; Welburn, 2011; Zinsstag et al., 2010). In light of the threats of emerging infectious diseases, bioterrorism and other environmental hazards, efforts to bridge the gap between human and animal health surveillance mechanisms, and to integrate efforts and data, are also increasing. Sentinel systems play a prominent role in many of these efforts (Rabinowitz et al., 2009; Scotch et al., 2009). For example, the Canary database (http:/canarydatabase.org) is a resource that continually collates evidence from scientific studies on the use of animals as early warning sentinels of human health hazards.

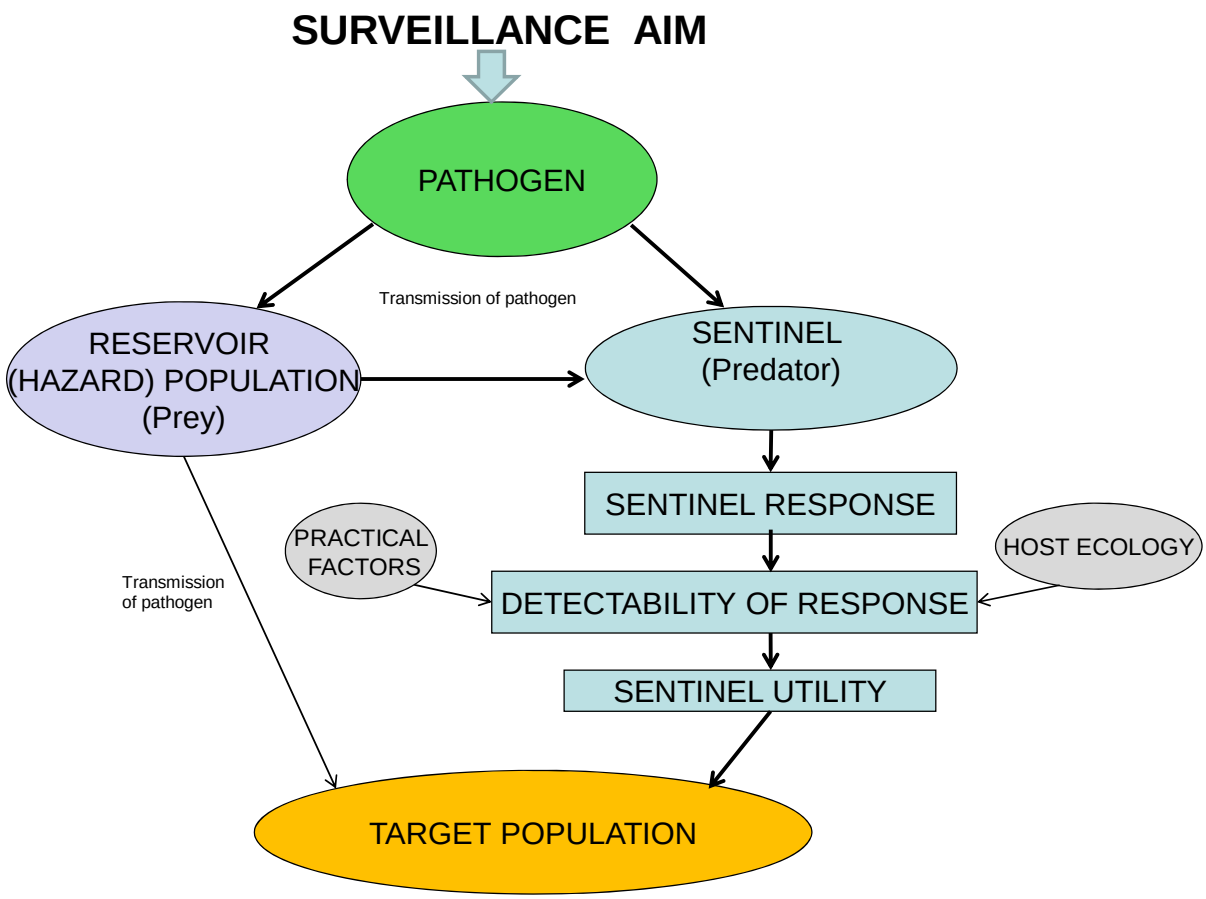
As discussed in Chapter 1, animal sentinels have the potential to be used to address several surveillance questions, including -

- Detection of a pathogen in an area
- Detection of changes of prevalence or incidence of a pathogen or disease over time
- Determination of the rate and direction of pathogen spread
- Testing specific hypotheses about pathogen ecology
- Evaluation of the effectiveness of disease control interventions (McCluskey, 2003).

However, prior to using sentinels, the surveillance question or aim must first be defined, only after which can a sentinel framework be established that considers the attributes of host species that need to be considered to identify an appropriate sentinel population. One conceptual sentinel framework proposed by Halliday et al (2007) (see Appendix 1) consists of the pathogen under surveillance, the target population, and the sentinel population. Under this framework, for the present study the target population would be defined as the prey. However, for the three pathogens investigated (*C. burnetii, Leptospira* spp and *E.cuniculi*), an important reason for selection was their zoonotic potential or risk to livestock. Therefore, in reality, the ultimate target, in terms of the output of the sentinel surveillance, would in fact be the human or livestock population to which the pathogen would be a potential hazard. In this type of surveillance situation the term target population is therefore misleading and the term "hazard population" or "pathogen reservoir population" would be more appropriate to describe the prey.

The term target population would be more applicable when it is the population at which surveillance efforts are directed, be that animal, (e.g. sentinel chickens in poultry flocks in surveillance for various avian diseases), or human, (e.g. patients seeking healthcare for influenza-like illnesses, or pyrexia detection at airports in surveillance for highly pathogenic influenza-A). In the present study, the selection of RHDV, although not investigated, would fit this criterion of a target population, as the risk is to the rabbit (prey) population only. Therefore, an adapted sentinel framework from that proposed by Halliday et al (2007) is presented in Figure 8.1 to better reflect the use of predators as sentinels in situations where the prey are not themselves the focus, or target, of surveillance.

Figure 8.1 A framework for predator as sentinels (adapted from Halliday et al 2007)



The sentinel response to a pathogen can vary, to include current infection (i.e. presence of the pathogen within the body), seroconversion, morbidity or mortality, and this will affect its detectability (Halliday et al., 2007). Using this modified framework (Figure 8.1) the selected response measured in this study was seroconversion. Practical factors encountered in this study included sample acquisition, sample size and diagnostic testing methodology, and relevant host ecology included predation habits and home range sizes, all of which may have influenced detection, as discussed in Chapter 7.

The study utilised the above framework to establish proof of principle that predators do have potential utility as sentinels, and in the next section these results are built on to consider some potential future means of how predators could be incorporated into surveillance programmes, particularly in the UK.

8.2 Means of incorporation of predators as sentinels into future surveillance programmes

The results of the present study indicate that the most useful application of predators in particular as sentinels is as a tool for general detection of pathogen presence or absence in a given area, rather than for detailed information about prevalence (Chapter 7). They also have the potential to provide information on the temporal patterns of pathogen presence, and effectively can act as a "memory-box" for many of the multiple pathogens they have been exposed to over their lifetime, especially if infection leads to long-lived seroconversion, and/or chronic infection or pathology. These two areas of application have also been suggested by others, for example from the findings that dogs and cats in Asia are seropositive for H5N1 avian influenza virus, and dogs for H5N1 and leptospirosis in Africa (Cleaveland et al., 2006; Halliday et al., 2007), and dogs in the USA for WNV (Resnick et al., 2008).

The selection of pathogen-prey-predator combinations in this study were as examples only for proof of principle, and not due to a particular identified surveillance need, but they can provide examples of where predators may be able to play a sentinel role as part of a surveillance programme. Animal sentinels may not be the most appropriate or useful surveillance tool in all circumstances, due to deficits in one or many of the key factors identified in the sentinel framework (e.g. sensitivity or specificity of sentinel response, practical factors, detectability of response). For example, when addressing surveillance for *C burnetii* in livestock in order to assess current levels, spread of infection, and zoonotic implications, it might be argued that, although it is of interest that *C. burnetii* can be detected in both prey and predators, direct monitoring of livestock or humans themselves is likely to be more appropriate, i.e. sampling the target population directly, as is commonly performed (Adesiyun et al., 2011; Bacci et al., 2011; Guatteo et al., 2011).

However, following the recent outbreaks of *C burnetii* in the Netherlands, more interest has also been focused at the rodent reservoirs around ruminant farms that may be capable of independent maintenance of infection (Reusken et al., 2011). Therefore surveillance of cats or foxes, rather than the rodents themselves, could also be considered as a means of detecting the presence or absence of infection in these

reservoirs in a more cost-effective way, to indicate at-risk herds or areas where infection is absent, and help to inform a more generalised programme of monitoring and intervention.

Sentinel surveillance also has a potential role in conservation and re-introduction or translocation programmes for endangered species, for example where knowledge of the presence of endemic pathogens that may affect naïve released animals can affect decision-making (IUCN, 1998) (Mathews et al., 2007). Common wild canids have already been identified as potentially useful sentinels for canine vector-borne diseases that may affect critically endangered canid species (Aguirre, 2009), essentially acting as proxy species in this context, but this concept could be extended to include pathogens affecting other, or multiple taxa, including those acquired from prey. For example, in the current Scottish Beaver Trial for reintroduction of beavers (www.scottishbeavers.org.uk), although the introduced beavers were health screened according to IUCN guidelines, screening of wild rodents in the release environment was not performed, largely due to cost limitations (G.Goodman personal communication). This is a situation where perhaps predator screening, for example of feral mink or foxes within the release site, could have been considered as a cost-effective means of detecting pathogen presence.

Sample acquisition

Easy and cost-effective acquisition of samples is key to predators being useful sentinels, and opportunities exist for both wild and domestic predators. As in this study, wild predators are most commonly acquired by lethal control.

Foxes and other wild canids

In the UK, the fox is the only native mammalian predator regularly controlled in a widespread fashion, although non-native mink are also targeted and could provide an opportunity where available. National surveys of shot foxes obtained for gamekeepers and landowners have previously been used to identify the prevalence of certain zoonotic parasites in the UK (*Echinococcus*, *Trichinella* and *Toxoplasma* (Smith et al., 2003) and for parasites posing a threat to domestic dogs

(*Angiostrongylus vasorum*) (Morgan et al., 2008), and also in other countries; for example the presence of *E.multilocularis* was recently detected for the first time in Sweden via routine surveillance of foxes (Osterman et al., 2011; Wahlstrom et al., 2011). Other canids and predator species have been used in other countries, e.g. coyotes in the USA for bovine tuberculosis (Berentsen et al., 2011). Therefore fox or other wild canid surveillance is a tried and tested means of surveillance for both endemic and new pathogen threats. This would require systems to be in place to facilitate and co-ordinate collection of carcases or samples, which may involve payment or other incentives to those controlling these species. For example, in the UK, the Food and Environment Research Agency (Fera) already has an established network for fox carcase collection (A. Tomlinson, personal communication) for disease investigation.

Wildlife hospitals and rehabilitation centres could also offer a resource and opportunity for opportunistic sampling of wild predator species, and have been recognised as a source of information of monitoring ecosystem health (Sleeman, 2011).These resources are perhaps more likely to have veterinary involvement and post-mortem examinations where samples could be collected, but again would require a co-ordinated approach to collection and submission, which currently does not exist in the UK. Indeed, the involvement of professionals such as veterinary surgeons and nurses does not necessarily improve sample acquisition, as exemplified by the poor compliance to cat sample submission by veterinary practices in the present study (see below). The limitations of data due to the bias in admissions to wildlife rehabilitation centres towards human-induced diseases must also be recognised (Sleeman, 2011).

Cats and dogs

Domestic cats have been identified in several studies as providing a link between human and animal health and provide further opportunities for use as sentinels (Scotch et al., 2009), including successful use for detection of *C. burnetii* (Marrie et al., 1988); and the present study), *Toxoplasma gondii* (Dubey and Weigel, 1996) and haemorrhagic fever (Xu et al., 1987). However, in the present study cat serum

sample collection was not efficient and better systems would need to be put in place. This limitation would also be likely to apply to acquiring domestic dog samples. Acquisition at source from the veterinary surgeon or technician taking the blood sample could be streamlined by the provision of postal packs containing equipment and a return envelope to submit samples, but this may not in itself increase compliance. Other alternatives for resolving poor compliance issues might include incentive schemes, or utilisation of situations where multiple dogs or cats are collected at foci such as at shows, rescue centres or neutering clinics, although the inherent potential biases in all these systems would have to be acknowledged. For example sampling in association with rabies vaccination campaigns has been suggested as a cost-effective means of acquiring dog samples in Africa and Asia (Cleaveland et al., 2006).

Another resource would be to utilise large diagnostic laboratories that receive multiple country-wide samples (blood, serum, faeces, tissues). This would eliminate any additional collection efforts at source. Small animal surveillance schemes such as the SAVSNET project, (University of Liverpool) have been established to provide scanning surveillance information on small animal diseases from both a wide range of veterinary practices and from diagnostic laboratories, and retention and additional testing of samples submitted could be used to monitor presence or prevalence of pathogens which may not be the original veterinary focus of interest relating to that individual, but could provide sentinel information from predation. However, funding for this scheme is currently uncertain beyond the pilot stage (A. Radford, personal communication).

In addition, in the UK, samples from dogs and cats entering the country under the PETS scheme, currently required to test for efficacy of rabies vaccination only, (http://www.defra.gov.uk/wildlife-pets/pets/travel/pets/) could also be submitted for testing of other non-endemic pathogens of interest, and are currently a wasted resource and opportunity. Legal issues of sample ownership, informed consent and sampling for experimental studies would have to be addressed and these will vary according to country.

Corvids and other avian predators

Corvids, although not eventually investigated in this study, do represent a potential sentinel resource that could be explored further. Like foxes, corvids have been tried and tested as sentinels, via their high morbidity and mortality rather than serological exposure, for WNV (Eidson et al., 2001a; Eidson et al., 2001b; Komar, 2001). For example, very high seroprevalence of *T.gondii* and *Neospora caninum* have been detected recently in the common raven (*Corvus corax*) in Spain for the first time, suggesting an important role for this species in the epidemiology of these two parasites of both human and animal significance (Molina-Lopez et al., 2011), which suggests that further investigation of corvid species is warranted for this and other pathogens. Corvid samples were easily obtained for this study and routine control is widespread, so this resource could be tapped into in the same way as for fox samples. Other avian predators such as raptors are frequently protected under law and routine acquisition is not possible, but there will be circumstances where opportunistic sampling may occur, such as in wildlife rehabilitation centres as discussed above.

Thus predator sample acquisition will require the establishment, or use of pre-existing, appropriate and co-ordinated networks according to the sentinel species selected, and may include wildlife professionals, veterinary professionals, diagnostic laboratories and animal welfare organisations. The extent of these networks necessarily depends on the surveillance question being asked and may range from a focal study area, as in present study, to national surveillance that would require governmental or other regulatory control. In the UK, the GB Wildlife Disease Surveillance Partnership has recently (2009) been established to provide a more co-ordinated approach to both scanning surveillance for wildlife diseases and targeted surveillance including for *Trichinella*, chronic wasting disease, European Bat Lyssavirus, West Nile Virus and Avian Influenza (http://vla.defra.gov.uk/reports/docs/rep_survrep_gbwsp.pdf), and this is one area where optimum use of wildlife predator sampling could be introduced, but similar UK schemes do not currently exist for domestic predators.

Other pathogens and sentinel response

This study investigated pathogens known to be acquired, at least in part, via ingestion of prey, and utilised the high position of predators in the trophic chain as effective samplers of their prey. However, ingestion is not an essential criteria for pathogen acquisition and predator species can act as sentinels for other pathogens transmitted by other routes, for example vector-borne diseases. For example, domestic dogs are exquisitely susceptible to *Leishmania* infections, and because of this morbidity have been identified as a suitable sentinel host for monitoring the spread of this emerging pathogen into non-endemic territories (Gramiccia, ; Ready, 2010). *Leishmania* has recently been detected in the UK in domestic dogs that have travelled abroad, particularly to Spain, under the PETS scheme (Shaw et al., 2008; Shaw et al., 2009) and is recognised as a significant potential zoonotic risk in the UK.

Examples of other pathogens that fitted at least some of the study criteria, but were not investigated, have been discussed in Chapter 2 (Table 2.1, repeated here as Table 8.1). These included some identified as posing a high risk as a threat to human health; for example, in addition to *C. burnetii*, *Campylobacter* and *T.gondii* have been prioritised in the Netherlands as posing the highest risk for emerging zoonoses (Havelaar et al., 2010), and predators (feral mink) have already been suggested as possible sentinels for *T.gondii* (Sepulveda et al., 2011).

Table 8.1 Other candidate pathogens suitable for investigation (repeat of Table 2.1)

Candidate pathogen	Candidate prey species	Candidate predator species	Main reason for rejection	References
Bacteria				
Salmonella spp	Rodents, birds	Foxes	Detection not reliant on serology	Kapperud, 1998; Kapperud,1983; Refsum, 2002; Handeland,2008; Wales, 2009
Campylobacter spp	Rodents, rabbits, birds	Dogs, cats, badgers	Detection not reliant on serology, no established links between prey and predator species	Meerburg, 2006; {Fernie, 1977; Fernie, 1976; Kwan, 2008; Luechtefeld, 1980; Kapperud, 1983; Palmgren, 1997 Waldenstrom, 2002 ; Colles, 2008; Hughes, 2009
Yersinia spp	Rodents, birds	Foxes	Detection not reliant on serology (*Y.enterocolitica* and *Y.pseudotuberculosis*. *Y. pestis* not present in UK and vector-borne	Kapperud, 1983; Kapperud, 1977; Kapperud, 1975; Kaneko, 1981; Kaneko, 1979; Servan, 1979; Shayegani, 1986; Fukushima, 1991; Nikolova, 2001; Nersesov, 1997;
Pasteurella multocida	Rabbits, birds	Raptors, cats, dogs	Ingestion of infected ducks reported for raptors but no reports in mammalian carnivores. Only reported in UK in wild brown rats, no reports in wild carnivores	Petersen, 2001; Samuel, 2005; Blanchong, 2006; Williams, 1987; Quan, 1986; Curtis, 1980; Webster, 1995
Protozoa and fungi				
Cryptosporidia (C.parvum, C.muris)	Rodents, rabbits,	Foxes, badgers, cats, shrews	Detection not reliant on serology. Wildlife believed to pose low zoonotic risk due to host-adapted genotypes	Fayer, 1986; Chalmers, 1997; Webster, 1995; Sturdee, 1999; Sturdee, 2003; Mtambo, 1991; Feng, 2010
Viruses				
Hantaviruses (e.g.Puumala virus)	Rodents, shrews	Unknown	No definitive evidence of infection in dogs, cats, coyotes. Puumala virus not detected in UK	Jonsson, 2010; Song, 2009; Malecki, 1998; McCaughey, 1996; Bennett, 2010
Cowpox	Rodents	Cats, foxes	Main route of infection is inoculation rather than ingestion	Boulanger, 1995; Boulanger, 1996; Bennett, 1997; Crouch, 1995; Chantrey, 1999 ; Hazel, 2000; Henning, 1995; Muller, 1996; Bennett, 1996
Bornavirus	Rodents, birds	Cats, dogs, foxes, shrews, corvids	No reports in UK in wild prey or predator species	Ludwig, 2000; Berg, 1998; Reeves, 1998; Weissenbock, Okamoto, 2002; Dauphin, 2001; Kinnunen, 2007; Hilbe, 2006;
West Nile Virus	Unknown	Corvids, Dogs	Not reported in UK. Already ongoing surveillance in UK	Komar, 2001; Eidson, 2001; Eidson, 2001; Resnick, 2008

Several of these candidate pathogens were rejected for this study because the major diagnostic methodology was not serological and instead relied on faecal analysis (*Salmonella, Campylobacter, Yersinia, Pasteurella, Cryptosporidium)*. However, this presents a different opportunity for potential sentinel use, as faecal collection can be achieved without the sentinel animal being present or the need for it to be sampled directly, e.g. collection of scats or droppings. This approach has already been adopted for detection of coproantigens to *E. multilocularis* in collected fox faeces in several countries including the UK, Sweden (see Foxes above) and the USA as an alternative to necropsy (Raoul et al., 2001).

In the UK, mycobacterial infections could provide examples of pathogens that could be worthy of investigation via a sentinel approach and those that may not. For example, the sentinel approach has been studied for *M. bovis* in wild deer in North America using coyotes and other large carnivores (Atwood et al., 2007), but in the UK there are no such predators that would kill and consume potentially infected deer. However, wild rabbits are believed to act as an important reservoir for *M. avium* subsp *paratuberculosis (*MAP*)*, the agent of Johnes' disease. MAP has also been detected in feral cats, foxes and other wild carnivores, and corvids (Daniels et al., 2003; Judge et al., 2006; Palmer et al., 2005) and so this pathogen could be of interest for exploring the sentinel surveillance approach.

Potentail candidate pathogens for surveillance that are not yet present or established in the UK but pose a high risk to humans, include vector-borne pathogens such as *Leishmania* mentioned above, and *Franciscella tularensis*, which is found mainly in rodents and rabbits (Nigrovic and Wingerter, 2008; Wobeser et al., 2009). However, the few surveys of foxes in areas where this pathogen is known to exist have yet to detect serological evidence of infection (Amundson and Yuill, 1981; Miller et al., 2000).

Although it was not possible to demonstrate proof of principle for the viral pathogen in this study, other studies have suggested that predators may also be of use as sentinels, such as dogs for WNV (Resnick et al., 2008) and cats and dogs for avian

influenza (Desvaux et al., 2009; Halliday et al., 2007). Surveillance for these two pathogens is already undertaken in wildlife by the GB Wildlife Disease Surveillance Partnership, but this focuses on wild birds, so there is the potential for predators that consume wild birds to also be utilised in a sentinel context. For example, although it has been shown that testing wild birds found dead is more effective and efficient than screening live wild birds in surveillance for H5N1 avian influenza (Knight-Jones et al., 2010), it may be that screening of predators that consume wild birds may also be a cost-effective option in determining presence or absence of this pathogen in an area. However, in this scenario there may be a time lag between introduction into an area and detection in the predators, which may not be desirable for certain surveillance aims where rapid detection of an outbreak and rapid response is required.

For the UK, the detection of hantaviruses in Northern Ireland and demonstration that conditions exist to permit cycling of Puumala virus in voles if it arrives in the UK (Bennett et al., 2010; McCaughey et al., 1996) suggest that hantaviruses might be a suitable candidate for further investigation of the sentinel approach. In addition, bornaviruses, having been detected in rodents, shrews and birds, as well as cats and foxes, and a suspected pathogen in man, again may offer a further opportunity for exploring sentinel surveillance using predators (Berg et al., 2001; Dauphin et al., 2001; Hilbe et al., 2006; Kinnunen et al., 2007; Reeves et al., 1998).

Diagnostic methods

New serological and diagnostic techniques could overcome many of the challenges posed by serological testing and test development, as found in the present study. For example, the EU WildTech project (EU 7th Framework Programme for Research and Technological Development), is currently developing nucleic acid and peptide based array techniques, and multiplex ELISA techniques, with the aim of allowing the high throughput screening of samples from a wide variety of wild animal species either at pathogen genus, species or strain level (www.wildtechproject.com). These techniques have great potential to streamline and reduce the cost of disease surveillance, but in turn are likely to raise new challenges if they also lead to

detection of novel pathogens whose significance to animal or human health is unknown (Halliday et al., 2011) and may require a sudden response.

It has been recognised that animal sentinels cannot provide the solution to the question of how to carry out surveillance for pathogens that are currently unknown (Halliday et al., 2007). However, perhaps as a consequence of greater awareness of the potential of animal sentinels and improved observation of animal populations, instances of unusual morbidity and mortality in animal populations that result from the emergence of novel pathogens may be more likely to be noticed and their potential significance to other species recognised.

In summary, for any surveillance situation, the context in which sentinels might be useful must be carefully identified, and ultimately how data will be used to address a defined surveillance aim and inform a defined response, such as implementation and evaluation of specific disease control interventions. As identified above, even though predators do have the ability to act as sentinels as indicated in this study, many practical and often political hurdles exist. These include collection and storage of, and access to, samples, incentives to submit samples, test methodology and validation, and not least funding of any surveillance scheme and co-ordination and integration of data at a local, regional or national level.

References

Adams LG (2001) In vivo and in vitro diagnosis of Mycobacterium bovis infection. Rev Sci Tech 20:304-324

Adesiyun A, Dookeran S, Stewart-Johnson A, Rahaman S, Bissessar S (2011) Frequency of seropositivity for Coxiella burnetii immunoglobulins in livestock and abattoir workers in Trinidad. New Microbiol 34:219-224

Adler B, de la Pena MA (2009) Leptospira and leptospirosis. Vet Microbiol

Adler B, Faine S, Gordon LM (1981) The enzyme-linked immunosorbent assay (ELISA) as a serological test for detecting antibodies against Leptospira interrogans serovar hardjo in sheep. Aust Vet J 57:414-417

Adler B, Murphy AM, Locarnini SA, Faine S (1980) Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. J Clin Microbiol 11:452-457

Agasi M, Oka C, Sato G (1967) Isolation of Salmonella typhimurium var Copenhagi from crows in the city of Otaru. Japanese Journal of Veterinary Science 38:521-522

Aguirre AA (2009) Wild canids as sentinels of ecological health: a conservation medicine perspective. Parasit Vectors 2 Suppl 1:S7:1-8

Agunloye CA, Nash AS (1996) Investigation of possible leptospiral infection in cats in Scotland. Journal of Small Animal Practice 37:126-129

Ahmad SN, Shah S, Ahmad FM (2005) Laboratory diagnosis of leptospirosis. J Postgrad Med 51:195-200

Akerstedt J (2002) An indirect ELISA for detection of Encephalitozoon cuniculi infection in farmed blue foxes (Alopex lagopus). Acta Vet Scand 43:211-220

Akerstedt J (2003) Humoral immune response in adult blue foxes (Alopex lagopus) after oral infection with Encephalitozoon cuniculi spores. Vet Parasitol 113:203-210

Akerstedt J, Kapel CM (2003) Survey for Encephalitozoon cuniculi in arctic foxes (Alopex lagopus) in Greenland. J Wildl Dis 39:228-232

Akerstedt J, Lillehaug A, Larsen IL, Eide NE, Arnemo JM, Handeland K (2010) Serosurvey for canine distemper virus, canine adenovirus, Leptospira interrogans, and Toxoplasma gondii in free-ranging canids in Scandinavia and Svalbard. J Wildl Dis 46:474-480

Akerstrom B, Brodin T, Reis K, Bjorck L (1985) Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. J Immunol 135:2589-2592

Alexander KA, Kat PW, House J, House C, O'Brien SJ, Laurenson MK, McNutt JW, Osburn BI (1995) African horse sickness and African carnivores. Vet Microbiol 47:133-140

Allela L, Bourry O, Pouillot R, Delicat A, Yaba P, Kumulungui B, Rouquet P, Gonzalez JP, Leroy EM (2005) Ebola virus antibody prevalence in dogs and human risk. Emerging Infect Dis 11:385-390

Amitai Z, Bromberg M, Bernstein M, Raveh D, Keysary A, David D, Pitlik S, Swerdlow D, Massung R, Rzotkiewicz S, Halutz O, Shohat T (2010) A large Q Fever outbreak in an urban school in central Israel. Clin Infect Dis 50:1433-1438

Amundson TE, Yuill TM (1981) Prevalence of selected pathogenic microbial agents in the red fox (Vulpes fulva) and gray fox (Urocyon cinereoargenteus) of southwestern Wisconsin. Journal of Wildlife Diseases 17:17-22

Anderson R.M., May RM (1992a) Infectious diseases of humans:dynamics and control. Oxford University Press, Oxford, UK

Anderson R.M., May RM (1992b) Biology of host-microparasite associations. Infectious diseases of humans: Dynamics and Control. Oxford University Press, Oxford, pp 27-65

Anderson DR, Burnham KP, White GC, Otis DL (1983) Density Estimation of Small-Mammal Populations Using a Trapping Web and Distance Sampling Methods. Ecology 64:674-680

Angelakis E, Raoult D (2010) Q Fever. Vet Microbiol 140:297-309

Anthony NM, Ribic CA, Bautz R, Garland T (2005) Comparative effectiveness of Longworth and Sherman live traps. Wildlife Society Bulletin 33:1018-1026

Arambulo P (2011) Veterinary public health in the age of "one health". J Am Vet Med Assoc 239:48-49

Artois, Bengis R, Delahay RJ, Duchene M, Duff JP, Ferroglio E, Gortazar C, Hutchings MR, Kock RA, Leighton FA, Morner T, Smith GC (2009) Wildlife Disease Surveillance and Monitoring. In: Delahay RJ, Smith GC, Hutchings MR (eds) Management of Disease in Wild Mammals. Springer, pp 187-213

Ashmawy KI, Abuakkada SS, Awad AM (2010) Seroprevalence of Antibodies to Encephalitozoon cuniculi and Toxoplasma gondii in Farmed Domestic Rabbits in Egypt. Zoonoses Public Health 58:357-364

Astobiza I, Barral M, Ruiz-Fons F, Barandika JF, Gerrikagoitia X, Hurtado A, Garcia-Perez AL (2010) Molecular investigation of the occurrence of Coxiella burnetii in wildlife and ticks in an endemic area. Vet Microbiol

Atwood TC, VerCauteren KC, DeLiberto TJ, Smith HJ (2007) Coyotes as sentinels for monitoring bovine tuberculosis prevalence in white-tailed deer. Journal of Wildlife Management 71:1545-1554

Augot D, Sauvage F, Boue F, Bouloy M, Artois M, Demerson JM, Combes B, Coudrier D, Zeller H, Cliquet F, Pontier D (2008) Spatial and temporal patterning of bank vole demography and the epidemiology of the Puumala hantavirus in northeastern France. Epidemiol Infect 136:1638-1643

Austgen LE, Bowen RA, Bunning ML, Davis BS, Mitchell CJ, Chang GJ (2004) Experimental infection of cats and dogs with West Nile virus. Emerg Infect Dis 10:82-86

Babudieri B (1958) Animal reservoirs of leptospires. Ann N Y Acad Sci 70:393-413

Babudieri B (1959) Q fever: a zoonosis. Advances in Veterinary Science and Comparative Medicine 5:81-181

Babudieri B, Carlos ER, Carlos ET, Jr. (1973) Pathogenic leptospira isolated from toad kidneys. Trop Geogr Med 25:297-299

Bacci S, Villumsen S, Valentiner-Branth P, Smith B, Krogfelt KA, Molbak K (2011) Epidemiology and Clinical Features of Human Infection with Coxiella burnetii in Denmark During 2006-07. Zoonoses Public Health doi:10.1111/j.1863-2378.2011.01419.x:

Backer LC, Grindem CB, Corbett WT, Cullins L, Hunter JL (2001) Pet dogs as sentinels for environmental contamination. Sci Total Environ 274:161-169

Bajani MD, Ashford DA, Bragg SL, Woods CW, Aye T, Spiegel RA, Plikaytis BD, Perkins BA, Phelan M, Levett PN, Weyant RS (2003) Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis. J Clin Microbiol 41:803-809

Baker P, Furlong M, Southern S, Harris S (2006) The potential impact of red fox Vulpes vulpes predation in agricultural landscapes in lowland Britain. Wildlife Biology 12:39-50

Baranowski E, Ruiz-Jarabo CM, Domingo E (2001) Evolution of cell recognition by viruses. Science 292:1102-1105

Barnes AM, Quan TJ, Beard ML, Maupin GO (1988) Plague in American Indians, 1956-1987. MMWR CDC Surveill Summ 37:11-16

Barratt DG (1997) Home Range Size, Habitat Utilisation and Movement Patterns of Suburban and Farm Cats Felis catus. Ecography 20:271-280

Bender JB, Shulman SA (2004) Reports of zoonotic disease outbreaks associated with animal exhibits and availability of recommendations for preventing zoonotic disease transmission from animals to people in such settings. J Am Vet Med Assoc 224:1105-1109

Bengis RG, Leighton FA, Fischer JR, Artois M, Morner T, Tate CM (2004) The role of wildlife in emerging and re-emerging zoonoses. Rev Sci Tech 23:497-511

Bengtson SA, Nilsson A, Rundgren S (1989) Population Structure and Dynamics of Wood Mouse Apodemus sylvaticus in Iceland. Holarctic Ecology 12:351-368

Benmayor R, Hodgson DJ, Perron GG, Buckling A (2009) Host mixing and disease emergence. Curr Biol 19:764-767

Bennett E, Clement J, Sansom P, Hall I, Leach S, Medlock JM (2010) Environmental and ecological potential for enzootic cycles of Puumala hantavirus in Great Britain. Epidemiology and Infection 138:91-98

Bennett M, Begon ME (1997) Virus zoonoses--a long-term overview. Comp Immunol Microbiol Infect Dis 20:101-109

Bercovich Z, Taaijke R, Bokhout BA (1990) Evaluation of an ELISA for the diagnosis of experimentally induced and naturally occurring Leptospira hardjo infections in cattle. Vet Microbiol 21:255-262

Berentsen AR, Dunbar MR, Johnson SR, Robbe-Austerman S, Martinez L, Jones RL (2011) Active use of coyotes (Canis latrans) to detect Bovine Tuberculosis in northeastern Michigan, USA. Vet Microbiol 151:126-132

Berg AL, Raid-Smith R, Larsson M, Bonnett B (1998) Case control study of feline Borna disease in Sweden. Vet Rec 142:715-717

Berg M, Johansson M, Montell H, Berg AL (2001) Wild birds as a possible natural reservoir of Borna disease virus. Epidemiol Infect 127:173-178

Berri M, Laroucau K, Rodolakis A (2000) The detection of Coxiella burnetii from ovine genital swabs, milk and fecal samples by the use of a single touchdown polymerase chain reaction. Vet Microbiol 72:285-293

Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E, Vinetz JM (2003) Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 3:757-771

Biek R, Ruth TK, Murphy KM, Anderson CR, Jr., Johnson M, DeSimone R, Gray R, Hornocker MG, Gillin CM, Poss M (2006) Factors associated with pathogen seroprevalence and infection in Rocky Mountain cougars. J Wildl Dis 42:606-615

Binder S, Levitt AM, Sacks JJ, Hughes JM (1999) Emerging infectious diseases: public health issues for the 21st century. Science 284:1311-1313

Binninger CE, Beecham JJ, Thomas LA, Winward LD (1980) A serologic survey for selected infectious diseases of black bears in Idaho. J Wildl Dis 16:423-430

Biswas D, Roy S, Vijayachari P, Sugunan AP, Natarajaseenivasan K, Sehgal SC (2005) Comparison of immunoreactive proteins of commonly circulating serogroups of Leptospira in Andaman Islands, India. Indian J Med Res 121:151-158

Bjorck L, Kronvall G (1984) Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. J Immunol 133:969-974

Black SS, Steinohrt LA, Bertucci DC, Rogers LB, Didier ES (1997) Encephalitozoon hellem in budgerigars (Melopsittacus undulatus). Vet Pathol 34:189-198

Blancou J, Aubert MF (1997) [Transmission of rabies virus: importance of the species barrier]. Bull Acad Natl Med 181:301-311

Blancou J, Chomel BB, Belotto A, Meslin FX (2005) Emerging or re-emerging bacterial zoonoses: factors of emergence, surveillance and control. Vet Res 36:507-522

Blehert DS, Hicks AC, Behr M, Meteyer CU, Berlowski-Zier BM, Buckles EL, Coleman JT, Darling SR, Gargas A, Niver R, Okoniewski JC, Rudd RJ, Stone WB (2009) Bat white-nose syndrome: an

emerging fungal pathogen? Science 323:227

Bomfim MR, Ko A, Koury MC (2005) Evaluation of the recombinant LipL32 in enzyme-linked immunosorbent assay for the serodiagnosis of bovine leptospirosis. Vet Microbiol 109:89-94

Boonstra R, Krebs CJ (1976) The effect of odour on trap response in Microtus townsendii. Journal of Zoology 180:467-476

Boot R, Hansen AK, Hansen CK, Nozari N, Thuis HC (2000) Comparison of assays for antibodies to Encephalitozoon cuniculi in rabbits. Lab Anim 34:281-289

Bossart GD (2006) Marine mammals as sentinel species for oceans and human health. Oceanography 19:134-137

Bradshaw JWS (1992) The behaviour of the domestic cat. C.A.B.I. Publishing, UK

Brahmbhatt (2005) Avian influenza: Economic and social impacts.

Brandao AP, Camargo ED, da Silva ED, Silva MV, Abrao RV (1998) Macroscopic agglutination test for rapid diagnosis of human leptospirosis. J Clin Microbiol 36:3138-3142

Brinkerhoff RJ, Collinge SK, Bai Y, Ray C (2009) Are carnivores universally good sentinels of plague? Vector Borne Zoonotic Dis 9:491-497

Bronsvoort BM, Parida S, Handel I, McFarland S, Fleming L, Hamblin P, Kock R (2008) Serological survey for foot-and-mouth disease virus in wildlife in eastern Africa and estimation of test parameters of a nonstructural protein enzyme-linked immunosorbent assay for buffalo. Clin Vaccine Immunol 15:1003-1011

Broom JC, Coghlan JD (1958) Leptospira ballum in small rodents in Scotland. Lancet 2:1041-1042

Brown C (2004) Emerging zoonoses and pathogens of public health significance--an overview. Rev Sci Tech 23:435-442

Brown JA, LeFebvre RB, Pan MJ (1991) Protein and antigen profiles of prevalent serovars of Leptospira interrogans. Infect Immun 59:1772-1777

Brown KP, Moller H, Innes J, Alterio N (1996) Calibration of tunnel tracking rates to estimate relative abundance of ship rats (Rattus rattus) and mice (Mus musculus) in a New Zealand forest. New Zealand Journal of Ecology 20:271-275

Brown M, Lappin MR, Brown JL, Munkhtsog B, Swanson WF (2005) Exploring the ecologic basis for extreme susceptibility of Pallas' cats (Otocolobus manul) to fatal toxoplasmosis. J Wildl Dis 41:691-700

Brownstein JS, Freifeld CC, Reis BY, Mandl KD (2008) Surveillance Sans Frontieres: Internet-based emerging infectious disease intelligence and the HealthMap project. PLoS Med 5:e151

Bryant JE, Crabtree MB, Nam VS, Yen NT, Duc HM, Miller BR (2005) Isolation of arboviruses from mosquitoes collected in northern Vietnam. Am J Trop Med Hyg 73:470-473

Buddle BM, de Lisle GW, McColl K, Collins BJ, Morrissy C, Westbury HA (1997) Response of the

North Island brown kiwi, Apteryx australis mantelli and the lesser short-tailed bat, Mystacina tuberculata to a measured dose of rabbit haemorrhagic disease virus. N Z Vet J 45:109-113

Burgdorfer W, Pickens EG, Newhouse VF, Lackman DB (1963) Isolation of Coxiella burnetii from rodents in western Montana. J Infect Dis 112:181-186

Burnet FM, Freeman M (1937) Experimental studies on the virus of "Q" fever. Medical Journal of Australia 2:299-305

Burr P, Snodgrass D (2004) Demystifying diagnostic testing:serology. In Practice 26:498-502

Burrell, Seibert FM (1916) Gases found in coal mines., 14 edn. Bureau of Mines, Department of the Interior, Washington, DC,

Burthe S, Telfer S, Begon M, Bennett M, Smith A, Lambin X (2008) Cowpox virus infection in natural field vole Microtus agrestis populations: significant negative impacts on survival. J Anim Ecol 77:110-119

Butler D (2006) Disease surveillance needs a revolution. Nature 440:6-7

Buyukmihci N, Bellhorn RW, Hunziker J, Clinton J (1977) Encephalitozoon (Nosema) infection of the cornea in a cat. Javma-Journal of the American Veterinary Medical Association 171:355-357

Byrnes EJ, III, Li W, Lewit Y, Ma H, Voelz K, Ren P, Carter DA, Chaturvedi V, Bildfell RJ, May RC, Heitman J (2010) Emergence and pathogenicity of highly virulent Cryptococcus gattii genotypes in the northwest United States. PLoS Pathog 6:e1000850

Cai HY, Hornby G, Key DW, Osuch MR, Maxie MG (2002) Preliminary study on differentiation of Leptospira grippotyphosa and Leptospira sejroe from other common pathogenic leptospiral serovars in canine urine by polymerase chain reaction assay. J Vet Diagn Invest 14:164-168

Cairns K, Brewer M, Lappin MR (2007) Prevalence of Coxiella burnetii DNA in vaginal and uterine samples from healthy cats of north-central Colorado. J Feline Med Surg 9:196-201

Cancellotti FM, Renzi M (1991) Epidemiology and current situation of viral haemorrhagic disease of rabbits and the European brown hare syndrome in Italy. Rev Sci Tech 10:409-422

Canning EU, Lom J (1986) The microsporidia of vertebrates. Academic Press, London, UK

Carcopino X, Raoult D, Bretelle F, Boubli L, Stein A (2009) Q Fever during pregnancy: a cause of poor fetal and maternal outcome. Ann N Y Acad Sci 1166:79-89

Centers for Disease Control and Prevention (2008) Manual for the Surveillance of Vaccine-Preventable Diseases.

Chappel RJ, Goris M, Palmer MF, Hartskeerl RA (2004) Impact of proficiency testing on results of the microscopic agglutination test for diagnosis of leptospirosis. J Clin Microbiol 42:5484-5488

Check E (2004) Health concerns prompt US review of exotic-pet trade. Nature 427:277

Cheemaa PS, Srivastava SK, Amutha R, Singh S, Singh H, Sandey M (2007) Detection of pathogenic leptospires in animals by PCR based on lipL21 and lipL32 genes. Indian J Exp Biol 45:568-573

Chitty D, Kempson DA (1949) Prebaiting Small Mammals and a New Design of Live Trap. Ecology 30:536-542

Cho HJ, Gale SP, Masri SA, Malkin KL (1989) Diagnostic specificity, sensitivity and cross-reactivity of an enzyme-linked immunosorbent assay for the detection of antibody against Leptospira interrogans serovars pomona, sejroe and hardjo in cattle. Can J Vet Res 53:285-289

Cho SN, Uhm JR, Kim JD (1992) Comparative analysis of lipopolysaccharide and lipid antigens of Leptospira interrogans serovars. Yonsei Med J 33:24-31

Chomel BB, Belotto A, Meslin FX (2007) Wildlife, exotic pets, and emerging zoonoses. Emerg Infect Dis 13:6-11

Chomel BB, Jay MT, Smith CR, Kass PH, Ryan CP, Barrett LR (1994) Serological surveillance of plague in dogs and cats, California, 1979-1991. Comp Immunol Microbiol Infect Dis 17:111-123

Chomel BB, Marano N (2009) Essential veterinary education in emerging infections, modes of introduction of exotic animals, zoonotic diseases, bioterrorism, implications for human and animal health and disease manifestation. Rev Sci Tech 28:559-565

Churcher PB, Lawton JH (1987) Predation by domestic cats in an English village. Journal of Zoology 212:439-455

Cleaveland, Haydon D, Taylor L (2007) Overviews of Pathogen Emergence: Which Pathogens Emerge, When and Why? pp 85-111

Cleaveland, Hess GR, Dobson AP, Laurenson MK, McCallum HI, Roberts MG, Woodroffe R (2002) The role of pathogens in biological conservation. In: Hudson PJ, Rizzoli A, Grenfell BT, Heesterbeek H, Dobson AP (eds) The Ecology of Wildlife Diseases. Oxford University Press, Oxford, pp 139-150

Cleaveland S, Laurenson MK, Taylor LH (2001) Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. Philos Trans R Soc Lond B Biol Sci 356:991-999

Cleaveland S, Meslin FX, Breiman R (2006) Dogs can play useful role as sentinel hosts for disease. Nature 440:605

Cole, Ellinghausen HC, Rubin HL (1980) Laboratory diagnosis of leptospirosis of domestic animals. Proceedings of the U.S. Animal Health Association, 83 edn pp 189-199

Cole JR, Jr., Sulzer CR, Pursell AR (1973) Improved microtechnique for the leptospiral microscopic agglutination test. Appl Microbiol 25:976-980

Coleman PG, Fevre EM, Cleaveland S (2004) Estimating the public health impact of rabies. Emerg Infect Dis 10:140-142

Committee on Animals as Monitors of Environmental Hazards (1991) Animals as sentinels of environmental health hazards. National Academy Press, Washington, DC,

Conrad PA, Miller MA, Kreuder C, James ER, Mazet J, Dabritz H, Jessup DA, Gulland F, Grigg ME

(2005) Transmission of Toxoplasma: clues from the study of sea otters as sentinels of Toxoplasma gondii flow into the marine environment. Int J Parasitol 35:1155-1168

Contesse P, Hegglin D, Gloor S, Bontadina F, Deplazes P (2004) The diet of urban foxes (Vulpes vulpes) and the availability of anthropogenic food in the city of Zurich, Switzerland. Mammalian Biology - Zeitschrift fur Saugetierkunde 69:81-95

Cordwell SJ (2008) Sequential extraction of proteins by chemical reagents. Methods Mol Biol 424:139-146

Cousins DV, Robertson GM (1986) Use of enzyme immunoassay in a serological survey of leptospirosis in sheep. Aust Vet J 63:36-39

Cousins DV, Robertson GM, Hustas L (1985) The use of the enzyme-linked immunosorbent assay (ELISA) to detect the IgM and IgG antibody response to Leptospira interrogans serovars hardjo, pomona and tarassovi in cattle. Vet Microbiol 10:439-450

Cousins DV, Robertson GM, Parkinson J, Richards RB (1991) Use of the enzyme linked immunosorbent assay (ELISA) to detect the IgM and IgG antibody response to Leptospira interrogans Serovar hardjo in pregnant ewes. Zentralbl Bakteriol 275:335-342

Cox FE (2001) Concomitant infections, parasites and immune responses. Parasitology 122 Suppl:S23-S38

Cox JC, Pye D, Edmonds JW, Shepherd R (1980) An investigation of Encephalitozoon cuniculi in the wild rabbit Oryctolagus cuniculus in Victoria, Australia. J Hyg (Lond) 84:295-300

Cox JC, Ross J (1980) A serological survey of Encephalitozoon cuniculi infection in the wild rabbit in England and Scotland. Res Vet Sci 28:396

Crabtree M, Sang R, Lutomiah J, Richardson J, Miller B (2009) Arbovirus surveillance of mosquitoes collected at sites of active Rift Valley fever virus transmission: Kenya, 2006-2007. J Med Entomol 46:961-964

Crawley MC (1970) Some population dynamics of the Bank vole, Clethrionomys glareolus and the Wood mouse, Apodemus sylvaticus in mixed woodland. Journal of Zoology 160:71-89

Cray C, Arcia G, Schneider R, Kelleher SA, Arheart KL (2009) Evaluation of the usefulness of an ELISA and protein electrophoresis in the diagnosis of Encephalitozoon cuniculi infection in rabbits. Am J Vet Res 70:478-482

Cross, Drewe J, Patrek V, Pearce G, Samuel MD, Delahay RJ (2009) Wildlife Population Structure and Parasite Transmission:Implications for Disease Management. In: Delahay RJ, Smith GC, Hutchings MR (eds) Management of Disease in Wild Mammals. Springer, Tokyo, pp 9-29

Csokai J, Fuchs-Baumgartinger A, Maass G (2010) Detection of Encephalitozoon cuniculi-infection (strain II) by PCR in a cat with anterior uveitis. Wiener Tierarztliche Monatsschrift210-215

Cumberland P, Everard CO, Levett PN (1999) Assessment of the efficacy of an IgM-elisa and microscopic agglutination test (MAT) in the diagnosis of acute leptospirosis. Am J Trop Med Hyg

61:731-734

Daniels MJ, Hutchings MR, Beard PM, Henderson D, Greig A, Stevenson K, Sharp JM (2003) Do non-ruminant wildlife pose a risk of paratuberculosis to domestic livestock and vice versa in Scotland? J Wildl Dis 39:10-15

Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife--threats to biodiversity and human health. Science 287:443-449

Daszak P (2009) A Call for Smart Surveillance: A Lesson Learned from H1N1. EcoHealth 6:1-2

Dauphin G, Legay V, Sailleau C, Smondack S, Hammoumi S, Zientara S (2001) Evidence of Borna disease virus genome detection in French domestic animals and in foxes (Vulpes vulpes). J Gen Virol 82:2199-2204

Davidson RK, Orpetveit I, Moller L, Kapel CM (2009) Serological detection of anti-Trichinella antibodies in wild foxes and experimentally infected farmed foxes in Norway. Vet Parasitol 163:93-100

Defra (2010) Comparisons with the 1967 FMD outbreak.

Dekker JJA, Stein A, nig IMA (2001) A spatial analysis of a population of red fox (Vulpes vulpes) in the Dutch coastal dune area. Journal of Zoology 255:505-510

Delahay RJ, Smith GC, Barlow AM, Walker N, Harris A, Clifton-Hadley RS, Cheeseman CL (2007) Bovine tuberculosis infection in wild mammals in the South-West region of England: a survey of prevalence and a semi-quantitative assessment of the relative risks to cattle. Vet J 173:287-301

Delibes-Mateos M, Fernandez de Simon J, Villafuerte R, Ferreras P (2008) Feeding responses of the red fox (Vulpes vulpes) to different wild rabbit (Oryctolagus cuniculus) densities:a regional approach. European Journal of Wildlife Research 54:71-78

Deplazes P, Mathis A, Baumgartner R, Tanner I, Weber R (1996) Immunologic and molecular characteristics of Encephalitozoon-like microsporidia isolated from humans and rabbits indicate that Encephalitozoon cuniculi is a zoonotic parasite. Clin Infect Dis 22:557-559

Derrick EH (1937) "Q" fever, a new fever entity:clinical features, diagnosis and laboratory investigation. Medical Journal of Australia 2:281-299

Desvaux S, Marx N, Ong S, Gaidet N, Hunt M, Manuguerra JC, Sorn S, Peiris M, Van der Werf S, Reynes JM (2009) Highly pathogenic avian influenza virus (H5N1) outbreak in captive wild birds and cats, Cambodia. Emerg Infect Dis 15:475-478

Dey S, Madhan MC, Ramadass P, Nachimuthu K (2007) Recombinant antigen-based latex agglutination test for rapid serodiagnosis of leptospirosis. Vet Res Commun 31:9-15

Dey S, Mohan CM, Kumar TM, Ramadass P, Nainar AM, Nachimuthu K (2004) Recombinant LipL32 antigen-based single serum dilution ELISA for detection of canine leptospirosis. Vet Microbiol 103:99-106

Didier ES (2005) Microsporidiosis: an emerging and opportunistic infection in humans and animals. Acta Trop 94:61-76

Didier ES, Visvesvara GS, Baker MD, Rogers LB, Bertucci DC, De Groote MA, Vossbrinck CR (1996) A microsporidian isolated from an AIDS patient corresponds to Encephalitozoon cuniculi III, originally isolated from domestic dogs. J Clin Microbiol 34:2835-2837

Didier ES, Vossbrinck CR, Baker MD, Rogers LB, Bertucci DC, Shadduck JA (1995) Identification and characterization of three Encephalitozoon cuniculi strains. Parasitology 111 (Pt 4):411-421

Diesch SL, McCulloch WF, Braun JL, Davis JR (1970) Detection and ecology of leptospirosis in Iowa wildlife. J Wildl Dis 6:275-288

Diesch SL, McCulloch WF, Braun JL, Ellinghausen HC, Jr. (1966) Leptospires isolated from frog kidneys. Nature 209:939-940

Dietz K (1993) The estimation of the basic reproduction number for infectious diseases. Stat Methods Med Res 2:23-41

Dipineto L, Rinaldi L, Santaniello A, Sensale M, Cuomo A, Calabria M, Menna LF, Fioretti A (2008) Serological survey for antibodies to Encephalitozoon cuniculi in pet rabbits in Italy. Zoonoses Public Health 55:173-175

Dobson A, Foufopoulos J (2001) Emerging infectious pathogens of wildlife. Philos Trans R Soc Lond B Biol Sci 356:1001-1012

Dohoo I, Martin W, Stryhn H (2003) Veterinary Epidemiological Research. University of Prince Edward Island, Charlottetown

Domingo E (2010) Mechanisms of viral emergence. Vet Res 41:38

Domingo E, Holland JJ (1997) RNA virus mutations and fitness for survival. Annu Rev Microbiol 51:151-178

Drewe JA, Dean GS, Michel AL, Lyashchenko KP, Greenwald R, Pearce GP (2009) Accuracy of three diagnostic tests for determining Mycobacterium bovis infection status in live-sampled wild meerkats (Suricata suricatta). J Vet Diagn Invest 21:31-39

Drummond MF, Sculpher MJ, Torrance GW, O'Brien BJ, Stoddart GL (2005) Methods for the economic evaluation of health care programmes, 3rd edn. Oxford University Press, Oxford

Dubey JP, Weigel RM (1996) Epidemiology of Toxoplasma gondii in farm ecosystems. J Eukaryot Microbiol 43:124S

Dugan VG, Yabsley MJ, Tate CM, Mead DG, Munderloh UG, Herron MJ, Stallknecht DE, Little SE, Davidson WR (2006) Evaluation of white-tailed deer (Odocoileus virginianus) as natural sentinels for Anaplasma phagocytophilum. Vector Borne Zoonotic Dis 6:192-207

Duncan AW, Correa MT, Levine JF, Breitschwerdt EB (2005) The dog as a sentinel for human infection: prevalence of Borrelia burgdorferi C6 antibodies in dogs from southeastern and mid-

Atlantic States. Vector Borne Zoonotic Dis 5:101-109

Dupont HT, Thirion X, Raoult D (1994) Q fever serology: cutoff determination for microimmunofluorescence. Clin Diagn Lab Immunol 1:189-196

Dyczkowski J, Yalden DW (1998) An estimate of the impact of predators on the British Field Vole Microtus agrestis population. Mammal Review 28:165-184

Dye, Williams BG (1995) Non-linearities in the dynamics of indirectly-transmitted infections (or, does having a vector make a difference?). In: Grenfell BT, Dobson AP (eds) Ecology of infectious diseases in natural populations. Cambridge University Press, Cambridge, pp 260-279

Eadie WR (1952) Shrew predation and vole populations in a localised area. Journal of Mammology 33:185-189

Eidson M, Komar N, Sorhage F, Nelson R, Talbot T, Mostashari F, McLean R (2001a) Crow deaths as a sentinel surveillance system for West Nile virus in the northeastern United States, 1999. Emerg Infect Dis 7:615-620

Eidson M, Kramer L, Stone W, Hagiwara Y, Schmit K (2001b) Dead bird surveillance as an early warning system for West Nile virus. Emerg Infect Dis 7:631-635

Eidson M, Miller J, Kramer L, Cherry B, Hagiwara Y (2001c) Dead crow densities and human cases of West Nile virus, New York State, 2000. Emerg Infect Dis 7:662-664

Ejercito CL, Cai L, Htwe KK, Taki M, Inoshima Y, Kondo T, Kano C, Abe S, Shirota K, Sugimoto T, . (1993) Serological evidence of Coxiella burnetii infection in wild animals in Japan. J Wildl Dis 29:481-484

Ellis WA, Songer JG, Montgomery J, Cassells JA (1986) Prevalence of Leptospira interrogans serovar hardjo in the genital and urinary tracts of non-pregnant cattle. Vet Rec 118:11-13

Enright JB, Franti CE, Behymer DE, Longhurst WM, Dutson VJ, Wright ME (1971) Coxiella burneti in a wildlife-livestock environment. Distribution of Q fever in wild mammals. Am J Epidemiol 94:79-90

Enserink M (2010) Infectious diseases. Questions abound in Q-fever explosion in the Netherlands. Science 327:266-267

Espinal MA (2003) The global situation of MDR-TB. Tuberculosis (Edinb) 83:44-51

Estrada-Franco JG, Bhatia V, az-Albiter H, Ochoa-Garcia L, Barbabosa A, Vazquez-Chagoyan JC, Martinez-Perez MA, Guzman-Bracho C, Garg N (2006) Human Trypanosoma cruzi infection and seropositivity in dogs, Mexico. Emerg Infect Dis 12:624-630

Faine S, Adler B, Palit A (1974) Chemical, serological and biological properties of a serotype-specific polysaccharide antigen in Leptospira. Aust J Exp Biol Med Sci 52:311-319

Ferguson DV, Heidt GA (1981) Survey for rabies, leptospirosis, toxoplasmosis and tularemia in striped skunks (Mephitis mephitis) from three public use areas in northwestern Arkansas. J Wildl Dis

17:515-519

Ferrari N (1995) Influence of the abundance of food resources on the feeding habits of the red fox, Vulpes vulpes, in western Switzerland. Journal of Zoology 236:117-129

Field PR, Mitchell JL, Santiago A, Dickeson DJ, Chan SW, Ho DW, Murphy AM, Cuzzubbo AJ, Devine PL (2000) Comparison of a commercial enzyme-linked immunosorbent assay with immunofluorescence and complement fixation tests for detection of Coxiella burnetii (Q fever) immunoglobulin M. J Clin Microbiol 38:1645-1647

Fiset P, Ormsbee RA, Silberman R, Peacock M, Spielman SH (1969) A microagglutination technique for detection and measurement of rickettsial antibodies. Acta Virol 13:60-66

Fitzgerald BM, Karl BJ (1986) Home range of feral house cats (Felis catus L.) in forest of the Orongorongo valley, Wellington, New Zealand. New Zealand Journal of Ecology 9:71-81

Flannery B, Costa D, Carvalho FP, Guerreiro H, Matsunaga J, da Silva ED, Ferreira AG, Riley LW, Reis MG, Haake DA, Ko AI (2001) Evaluation of recombinant Leptospira antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. J Clin Microbiol 39:3303-3310

Fleming WJ, Nusbaum SR, Caslick JW (1979) Serologic evidence of leptospirosis in woodchucks (Marmota monax) in central New York State. J Wildl Dis 15:245-251

Flowerdew JR (1985) The population dynamics of wood mice and yellow-necked mice. Symposia of the Zoological Society of London 55:315-338

Flowerdew JR (1976) Chapter 4:Ecological methods. Mammal Review 6:123-159

Forrester NL, Boag B, Buckley A, Moureau G, Gould EA (2009) Co-circulation of widely disparate strains of rabbit haemorrhagic disease virus could explain localised epidemicity in the United Kingdom. Virology 393:42-48

Fournier PE, Marrie TJ, Raoult D (1998) Diagnosis of Q fever. J Clin Microbiol 36:1823-1834

Fournier S, Liguory O, Sarfati C, vid-Ouaknine F, Derouin F, Decazes JM, Molina JM (2000) Disseminated infection due to Encephalitozoon cuniculi in a patient with AIDS: case report and review. HIV Med 1:155-161

Frangoulidis D, Schropfer E, Al DS, Tomaso H, Meyer H (2006) Comparison of four commercially available assays for the detection of IgM phase II antibodies to Coxiella burnetii in the diagnosis of acute Q fever. Ann N Y Acad Sci 1078:561-562

Franzen C (2005) How do microsporidia invade cells? Folia Parasitol (Praha) 52:36-40

Franzen C, Muller A, Hartmann P, Hegener P, Schrappe M, Diehl V, Fatkenheuer G, Salzberger B (1998) Polymerase chain reaction for diagnosis and species differentiation of microsporidia. Folia Parasitol (Praha) 45:140-148

Frolich K, Klima F, Dedek J (1998) Antibodies against rabbit hemorrhagic disease virus in free-ranging red foxes from Germany. J Wildl Dis 34:436-442

Fuller HE, Chasey D, Lucas MH, Gibbens JC (1993) Rabbit haemorrhagic disease in the United Kingdom. Vet Rec 133:611-613

Gale P, Brouwer A, Ramnial V, Kelly L, Kosmider R, Fooks AR, Snary EL (2010) Assessing the impact of climate change on vector-borne viruses in the EU through the elicitation of expert opinion. Epidemiol Infect 138:214-225

Ganapathy K, Saleha AA, Jaganathan M, Tan CG, Chong CT, Tang SC, Ideris A, Dare CM, Bradbury JM (2007) Survey of campylobacter, salmonella and mycoplasmas in house crows (Corvus splendens) in Malaysia. Vet Rec 160:622-624

Gannon J (1980) A survey of Encephalitozoon cuniculi in laboratory animal colonies in the United Kingdom. Lab Anim 14:91-94

Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, Michael SF, Cummins LB, Arthur LO, Peeters M, Shaw GM, Sharp PM, Hahn BH (1999) Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature 397:436-441

Garamszegi LZ (2009) Patterns of co-speciation and host switching in primate malaria parasites. Malar J 8:110

Garcia-Bocanegra I, Dubey JP, Martinez F, Vargas A, Cabezon O, Zorrilla I, Arenas A, Almeria S (2010) Factors affecting seroprevalence of Toxoplasma gondii in the endangered Iberian lynx (Lynx pardinus). Vet Parasitol 167:36-42

Gardner IA, Hietala S, Boyce WM (1996) Validity of using serological tests for diagnosis of diseases in wild animals. Rev Sci Tech 15:323-335

Georges-Courbot MC, Sanchez A, Lu CY, Baize S, Leroy E, Lansout-Soukate J, Tevi-Benissan C, Georges AJ, Trappier SG, Zaki SR, Swanepoel R, Leman PA, Rollin PE, Peters CJ, Nichol ST, Ksiazek TG (1997) Isolation and phylogenetic characterization of Ebola viruses causing different outbreaks in Gabon. Emerg Infect Dis 3:59-62

Gerritsen MJ, Koopmans MJ, Olyhoek T (1993) Effect of streptomycin treatment on the shedding of and the serologic responses to Leptospira interrogans serovar hardjo subtype hardjobovis in experimentally infected cows. Vet Microbiol 38:129-135

Gilbert L (2010) Altitudinal patterns of tick and host abundance: a potential role for climate change in regulating tick-borne diseases? Oecologia 162:217-225

Giminez DF (1964) Staining rickettsia in cell cutures. Stain Technol 39:135-140

Ginsberg JR, Mace GM, Albon S (1995) Local extinction in a small and declining population: wild dogs in the Serengeti. Proc Biol Sci 262:221-228

Goldstein T, Mazet JA, Gill VA, Doroff AM, Burek KA, Hammond JA (2009) Phocine distemper virus in northern sea otters in the Pacific Ocean, Alaska, USA. Emerg Infect Dis 15:925-927

Goodwin D, Gennari SM, Howe DK, Dubey JP, Zajac AM, Lindsay DS (2006) Prevalence of antibodies to Encephalitozoon cuniculi in horses from Brazil. Vet Parasitol 142:380-382

Goszczynski (2002) Home ranges in red fox: territoriality diminishes with increasing area., 47 edn pp 103-114

Graham RL, Baric RS (2010) Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. J Virol 84:3134-3146

Gramiccia M Recent advances in leishmaniosis in pet animals: Epidemiology, diagnostics and anti-vectorial prophylaxis. Veterinary Parasitology In Press, Corrected Proof:

Gravekamp C, Korver H, Montgomery J, Everard CO, Carrington D, Ellis WA, Terpstra WJ (1991) Leptospires isolated from toads and frogs on the Island of Barbados. Zentralbl Bakteriol 275:403-411

Greger M (2007) The human/animal interface: emergence and resurgence of zoonotic infectious diseases. Crit Rev Microbiol 33:243-299

Greiner M, Franke CR, Bohning D, Schlattmann P (1994) Construction of an intrinsic cut-off value for the sero-epidemiological study of Trypanosoma evansi infections in a canine population in Brazil: a new approach towards an unbiased estimation of prevalence. Acta Trop 56:97-109

Greiner M, Gardner IA (2000) Epidemiologic issues in the validation of veterinary diagnostic tests. Preventive Veterinary Medicine 45:3-22

Groseth A, Feldmann H, Strong JE (2007) The ecology of Ebola virus. Trends Microbiol 15:408-416

Guatteo R, Seegers H, Taurel AF, Joly A, Beaudeau F (2011) Prevalence of Coxiella burnetii infection in domestic ruminants: a critical review. Vet Microbiol 149:1-16

Guerreiro H, Croda J, Flannery B, Mazel M, Matsunaga J, Galvao RM, Levett PN, Ko AI, Haake DA (2001) Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect Immun 69:4958-4968

Gurnell J (1976) Studies on the effect of bait and sampling intensity on trapping and estimating wood mice Apodemus sylvaticus. Journal of Zoology 171:458-464

Gurnell, Flowerdew JR (2006) Live Trapping Small Mammals: A Practical Guide. The Mammal Society,

Gurnell J, Langbein J (1983) Effects of trap position on the capture of woodland rodents. Journal of Zoology 217:241-254

Gurnell J, Little J (1992) The influence of trap residual odour on catches of woodland rodents. Animal Behaviour 42:623-632

Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, Matsunaga J, Levett PN, Bolin CA (2000) The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect Immun 68:2276-2285

Hackstadt T (1990) The role of lipopolysaccharides in the virulence of Coxiella burnetii. Ann N Y Acad Sci 590:27-32

Halanova M, Cislakova L, Valencakova A, Balent P, Adam J, Travnicek M (2003) Serological

screening of occurrence of antibodies to Encephalitozoon cuniculi in humans and animals in Eastern Slovakia. Annals of Agricultural and Environmental Medicine 10:117-120

Halliday J, Daborn C, Auty H, Hampson K, Mtema Z, Knobel D, Cleaveland S (2011) Gaps in global surveillance for emerging zoonoses:why it makes sens to focus on endemic zoonoses. Phil Trans R Soc Lond Biol (Submitted):

Halliday (2010) Animal sentinel surveillance: evaluating domestic dogs as sentinels for zoonotic pathogen surveillance. University of Edinburgh,

Halliday JE, Meredith AL, Knobel DL, Shaw DJ, Bronsvoort BM, Cleaveland S (2007) A framework for evaluating animals as sentinels for infectious disease surveillance. J R Soc Interface 4:973-984

Hamilton CM, Gray R, Wright SE, Gangadharan B, Laurenson K, Innes EA (2005) Prevalence of antibodies to Toxoplasmagondii and Neosporacaninum in red foxes (Vulpesvulpes) from around the UK. Vet Parasitol 130:169-173

Harcourt-Brown FM, Holloway HK (2003) Encephalitozoon cuniculi in pet rabbits. Vet Rec 152:427-431

Harkin KR, Roshto YM, Sullivan JT, Purvis TJ, Chengappa MM (2003) Comparison of polymerase chain reaction assay, bacteriologic culture, and serologic testing in assessment of prevalence of urinary shedding of leptospires in dogs. J Am Vet Med Assoc 222:1230-1233

Harris S (1978) Age determination in the red fox (Vulpes vulpes) - an evaluationof technique efficiency as applied to a sample of suburban foxes. Journal of Zoology 184:91-117

Harris S (1981) The food of suburban foxes (Vulpes vulpes) wih special reference to London. Mammal Review 11:151-168

Harris, Morris P, Wray S, Yaldon D (1995) A Review of British Mammals. Joint Nature Conservation Committee, Peterborough,

Harris S, Yaldon DW (2008) Mammals of the British Isles: Handbook, 4th edn. The Mammal Society, Southampton

Hart CA, Bennett M, Begon ME (1999) Zoonoses. Journal of Epidemiology and Community Health 53:514-515

Hartman EG, van d, I, Rothuizen J (1986) Clinical, pathological and serological features of spontaneous canine leptospirosis. An evaluation of the IgM- and IgG-specific ELISA. Vet Immunol Immunopathol 13:261-271

Hartman EG, van HM, Frik JF, van der Donk JA (1984a) Humoral immune response of dogs after vaccination against leptospirosis measured by an IgM- and IgG-specific ELISA. Vet Immunol Immunopathol 7:245-254

Hartman EG, van HM, van der Donk JA, Frik JF (1984b) Determination of specific anti-leptospiral immunoglobulins M and G in sera of experimentally infected dogs by solid-phase enzyme-linked immunosorbent assay. Vet Immunol Immunopathol 7:43-51

Hartman EG, van HM, van der Donk JA, Frik JF (1984c) Serodiagnosis of canine leptospirosis by solid-phase enzyme-linked immunosorbent assay. Vet Immunol Immunopathol 7:33-42

Hathaway SC, Little TW, Headlam SA, Stevens AE (1983a) Infection of free-living carnivores with leptospires of the Australis serogroup. Vet Rec 113:233-235

Hathaway SC, Little TW, Stevens AE, Ellis WA, Morgan J (1983b) Serovar identification of leptospires of the Australis serogroup isolated from free-living and domestic species in the United Kingdom. Res Vet Sci 35:64-68

Havelaar AH, van RF, Bucura C, Toetenel MA, Haagsma JA, Kurowicka D, Heesterbeek JH, Speybroeck N, Langelaar MF, van der Giessen JW, Cooke RM, Braks MA (2010) Prioritizing emerging zoonoses in the Netherlands. PLoS One 5:e13965

Haydon DT, Chase-Topping M, Shaw DJ, Matthews L, Friar JK, Wilesmith J, Woolhouse ME (2003) The construction and analysis of epidemic trees with reference to the 2001 UK foot-and-mouth outbreak. Proc Biol Sci 270:121-127

Hayes EB, Gubler DJ (2006) West Nile virus: epidemiology and clinical features of an emerging epidemic in the United States. Annu Rev Med 57:181-194

Hazel SM, Bennett M, Chantrey J, Bown K, Cavanagh R, Jones TR, Baxby D, Begon M (2000) A longitudinal study of an endemic disease in its wildlife reservoir: cowpox and wild rodents. Epidemiol Infect 124:551-562

Henning K, Czerny CP, Meyer H, Muller T, Kramer M (1995) A seroepidemiological survey for orthopox virus in the red fox (Vulpes vulpes). Vet Microbiol 43:251-259

Hersteinsson P, Gunnarsson E, Hjartardottir S, Skirnisson K (1993) Prevalence of Encephalitozoon cuniculi antibodies in terrestrial mammals in Iceland, 1986 to 1989. J Wildl Dis 29:341-344

Heydon MJ, Reynolds JC, Short MJ (2000) Variation in abundance of foxes (Vulpes vulpes) between three regions of rural Britain, in relation to landscape and other variables. Journal of Zoology 251:253-264

Heydon MJ, Reynolds JC (2000) Demography of rural foxes (Vulpes vulpes) in relation to cull intensity in three contrasting regions of Britain. Journal of Zoology 251:265-276

Heyman P, Mele RV, Smajlovic L, Dobly A, Cochez C, Vandenvelde C (2009) Association between habitat and prevalence of hantavirus infections in bank voles (Myodes glareolus) and wood mice (Apodemus sylvaticus). Vector Borne Zoonotic Dis 9:141-146

Higgins R (2004) Emerging or re-emerging bacterial zoonotic diseases: bartonellosis, leptospirosis, Lyme borreliosis, plague. Rev Sci Tech 23:569-581

Hilbe M, Herrsche R, Kolodziejek J, Nowotny N, Zlinszky K, Ehrensperger F (2006) Shrews as reservoir hosts of borna disease virus. Emerg Infect Dis 12:675-677

Hirsch VM, Olmsted RA, Murphey-Corb M, Purcell RH, Johnson PR (1989) An African primate lentivirus (SIVsm) closely related to HIV-2. Nature 339:389-392

Hollander M, Wolfe D.A (1999) Nonparametric Statistical Methods, 2nd edn. John Wiley & Sons Inc., New York

Hollister WS, Canning EU, Viney M (1989) Prevalence of antibodies to Encephalitozoon cuniculi in stray dogs as determined by an ELISA. Vet Rec 124:332-336

Holmes EC, Drummond AJ (2007) The evolutionary genetics of viral emergence. Curr Top Microbiol Immunol 315:51-66

House C, Alexander KA, Kat PW, O'Brien SJ, Mangiafico J (1996) Serum antibody to Rift Valley fever virus in African carnivores. Ann N Y Acad Sci 791:345-349

Hsu V, Grant DC, Zajac AM, Witonsky SG, Lindsay DS (2011) Prevalence of IgG antibodies to Encephalitozoon cuniculi and Toxoplasma gondii in cats with and without chronic kidney disease from Virginia. Veterinary Parasitology 176:23-26

Hurkova L, Modry D (2006) PCR detection of Neospora caninum, Toxoplasma gondii and Encephalitozoon cuniculi in brains of wild carnivores. Vet Parasitol 137:150-154

Igarashi M, Oohashi E, Dautu G, Ueno A, Kariya T, Furuya K (2008) High seroprevalence of Encephalitozoon cuniculi in pet rabbits in Japan. J Vet Med Sci 70:1301-1304

Inganas M, Johansson SG, Sjoquist J (1981) Further characterization of the alternative protein-A interaction of immunoglobulins: demonstration of an Fc-binding fragment of protein A expressing the alternative reactivity. Scand J Immunol 14:379-388

Innes DGL, Bendall JF (1988) Sampling of small mammals by different types of traps in Northern Ontario, Canada. Acta Theriologica 33:443-450

Institute of Medicine (1992) Emerging infections:microbial threats to health in the United States. National Academy Press, Washington DC

International Committee on Systemic Bacteriology (1984) Subcommittee on the taxonomy of Leptospira. International Journal of Systematic Bacteriology 34:258-259

Invitrogen (2011)

IUCN (1998) Guideline for re-introductions. IUCN Publications, Cambridge

Jacob J, Ylanen H, Hodkinson C (2002) Trapping success of Ugglan traps and Longworth traps for house mice in South-Eastern Australia. Wildlife Research 29:101-103

Jeklova E, Jekl V, Kovarcik K, Hauptman K, Koudela B, Neumayerova H, Knotek Z, Faldyna M (2010) Usefulness of detection of specific IgM and IgG antibodies for diagnosis of clinical encephalitozoonosis in pet rabbits. Vet Parasitol 170:143-148

Johnson GD, Eidson M, Schmit K, Ellis A, Kulldorff M (2006) Geographic prediction of human onset of West Nile virus using dead crow clusters: an evaluation of year 2002 data in New York State. Am J Epidemiol 163:171-180

Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, Daszak P (2008) Global trends

in emerging infectious diseases. Nature 451:990-993

Jordan CN, Zajac AM, Snowden KS, Lindsay DS (2006) Direct agglutination test for Encephalitozoon cuniculi. Vet Parasitol 135:235-240

Joss AW, Mavin S, Ho-Yen DO (2007) Increased incidence of Lyme borreliosis following mild winters and during warm, humid summers. Eur J Clin Microbiol Infect Dis 26:79

Jost BH, Adler B, Vinh T, Faine S (1986) A monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. J Med Microbiol 22:269-275

Judge J, Kyriazakis I, Greig A, Davidson RS, Hutchings MR (2006) Routes of intraspecies transmission of Mycobacterium avium subsp. paratuberculosis in rabbits (Oryctolagus cuniculus): a field study. Appl Environ Microbiol 72:398-403

Julian KG, Eidson M, Kipp AM, Weiss E, Petersen LR, Miller JR, Hinten SR, Marfin AA (2002) Early season crow mortality as a sentinel for West Nile virus disease in humans, northeastern United States. Vector Borne Zoonotic Dis 2:145-155

Kaneene JB, Miller R, Meyer RM (2006) Abattoir surveillance: The U.S. experience. Veterinary Microbiology 112:273-282

Kaplan B (2011) One Health or... some health? Vet Ital 47:129-131

Kapperud G, Rosef O (1983) Avian wildlife reservoir of Campylobacter fetus subsp. jejuni, Yersinia spp., and Salmonella spp. in Norway. Appl Environ Microbiol 45:375-380

Karesh WB, Cook RA, Bennett EL, Newcomb J (2005) Wildlife trade and global disease emergence. Emerg Infect Dis 11:1000-1002

Kasickova D, Sak B, Kvac M, Ditrich O (2007) Detection of Encephalitozoon cuniculi in a new host--cockateel (Nymphicus hollandicus) using molecular methods. Parasitol Res 101:1685-1688

Katinka MD, Duprat S, Cornillot E, Metenier G, Thomarat F, Prensier G, Barbe V, Peyretaillade E, Brottier P, Wincker P, Delbac F, El AH, Peyret P, Saurin W, Gouy M, Weissenbach J, Vivares CP (2001) Genome sequence and gene compaction of the eukaryote parasite Encephalitozoon cuniculi. Nature 414:450-453

Katzwinkel-Wladarsch S, Deplazes P, Weber R, Loscher T, Rinder H (1997) Comparison of polymerase chain reaction with light microscopy for detection of microsporidia in clinical specimens. Eur J Clin Microbiol Infect Dis 16:7-10

Kazar J, Brezina R, Schramek S, Palanova A, Tvrda B (1981) Suitability of the microagglutination test for detection of post-infection and post-vaccination Q fever antibodies in human sera. Acta Virol 25:235-240

Keeble EJ, Shaw DJ (2006) Seroprevalence of antibodies to Encephalitozoon cuniculi in domestic rabbits in the United Kingdom. Vet Rec 158:539-544

Keeling PJ, Luker MA, Palmer JD (2000) Evidence from Beta-Tubulin Phylogeny that Microsporidia

Evolved from Within the Fungi. Mol Biol Evol 17:23-31

Kermack WO, McKendrick A.G (1927) A contribution to the mathematical theory of epidemics. Proc Roy Soc Lond 115:700-721

Kerr (1989) Clinical biochemistry. In: Doxey DLNMBF (ed) BSAVA Manual of Laboratory Techniques. BSAVA, Gloucester, p 25

Khan MA, Goyal SM, Diesch SL, Mech LD, Fritts SH (1991) Seroepidemiology of leptospirosis in Minnesota wolves. J Wildl Dis 27:248-253

Kidawa D, Kowalczyk R (2010) The effetcs of sex,age,season and habitat on diet of teh red fox Vulpes vulpes in northeastern Poland. Acta Theriologica

Kile JC, Fleischauer AT, Beard B, Kuehnert MJ, Kanwal RS, Pontones P, Messersmith HJ, Teclaw R, Karem KL, Braden ZH, Damon I, Khan AS, Fischer M (2005) Transmission of monkeypox among persons exposed to infected prairie dogs in Indiana in 2003. Arch Pediatr Adolesc Med 159:1022-1025

King DA, Peckham C, Waage JK, Brownlie J, Woolhouse ME (2006) Epidemiology. Infectious diseases: preparing for the future. Science 313:1392-1393

Kingscote BF (1986) Leptospirosis in red foxes in Ontario. J Wildl Dis 22:475-478

Kinnunen PM, Billich C, Ek-Kommonen C, Henttonen H, Kallio RK, Niemimaa J, Palva A, Staeheli P, Vaheri A, Vapalahti O (2007) Serological evidence for Borna disease virus infection in humans, wild rodents and other vertebrates in Finland. J Clin Virol 38:64-69

Klein SL, Calisher CH (2007) Emergence and persistence of hantaviruses. Curr Top Microbiol Immunol 315:217-252

Knight-Jones TJ, HAuser R, Matthes D, Stark KD (2010) Evaluation of effectiveness and efficiancy of wild bird surveillance for avian influenza. Vet Res 41:50:

Koch A, Svendsen CB, Christensen JJ, Bundgaard H, Vindfeld L, Christiansen CB, Kemp M, Villumsen S (2010) Q fever in Greenland. Emerg Infect Dis 16:511-513

Kock RA, Wambua JM, Mwanzia J, Wamwayi H, Ndungu EK, Barrett T, Kock ND, Rossiter PB (1999) Rinderpest epidemic in wild ruminants in Kenya 1993-97. Vet Rec 145:275-283

Kodjikian L, Garweg JG, Nguyen M, Schaffner T, Deplazese P, Zimmerli S (2005) Intraocular microsporidiosis due to Encephalitozoon cuniculi in a patient with idiopathic CD4+ T-lymphocytopenia. Int J Med Microbiol 294:529-533

Komar N (2001) West Nile virus surveillance using sentinel birds. Ann N Y Acad Sci 951:58-73

Komar N, Langevin S, Hinten S, Nemeth N, Edwards E, Hettler D, Davis B, Bowen R, Bunning M (2003) Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg Infect Dis 9:311-322

Komiya T, Sadamasu K, Kang M, Tsuboshima S, Fukushi H, Hirai K (2003a) Seroprevalence of

Coxiella burnetii infections among cats in different living environments. Journal of Veterinary Medical Science 65:1047-1048

Komiya T, Sadamasu K, Toriniwa H, Kato K, Arashima Y, Fukushi H, Hirai K, Arakawa Y (2003b) Epidemiological survey on the route of Coxiella burnetii infection in an animal hospital. J Infect Chemother 9:151-155

Korpimaki E, Norrdahl K, Huitu O, Klemola T (2005) Predator-induced synchrony in population oscillations of coexisting small mammal species. Proc Biol Sci 272:193-202

Kosatsky T (1984) Household outbreak of Q-fever pneumonia related to a parturient cat. Lancet 2:1447-1449

Kositanont U, Naigowit P, Imvithaya A, Singchai C, Puthavathana P (2003) Prevalence of antibodies to Leptospira serovars in rodents and shrews trapped in low and high endemic areas in Thailand. J Med Assoc Thai 86:136-142

Krebs CJ, Gaines MS, Keller BL, Myers JH, Tamarin RH (1973) Population cycles in small rodents. Science 179:35-41

Kronvall G, Seal US, Svensson S, Williams RC, Jr. (1974) Phylogenetic aspects of staphylococcal protein A-reactive serum globulins in birds and mammals. Acta Pathol Microbiol Scand B Microbiol Immunol 82:12-18

Kruse H, kirkemo AM, Handeland K (2004) Wildlife as source of zoonotic infections. Emerg Infect Dis 10:2067-2072

Kruszewska D, Tylewska-Wierzbanowska S (1997) Isolation of Coxiella burnetii from bull semen. Res Vet Sci 62:299-300

Kuiken T, Fouchier R, Rimmelzwaan G, Osterhaus A (2003) Emerging viral infections in a rapidly changing world. Curr Opin Biotechnol 14:641-646

Kuiken T, Leighton FA, Fouchier RA, LeDuc JW, Peiris JS, Schudel A, Stohr K, Osterhaus AD (2005) Public health. Pathogen surveillance in animals. Science 309:1680-1681

Kunstyr I, Lev L, Naumann S (1986) Humoral antibody response of rabbits to experimental infection with Encephalitozoon cuniculi. Vet Parasitol 21:223-232

Kunzel F, Joachim A (2010) Encephalitozoonosis in rabbits. Parasitol Res 106:299-309

Lambin X, Bretagnolle V, Yoccoz NG (2006) Vole population cycles in northern and southern Europe: is there a need for different explanations for single pattern? J Anim Ecol 75:340-349

Lambin X, MacKinnon J (1997) The relative efficiency of two commercial live-traps for small mammals. Journal of Zoology 242:400-404

Lambin X, Petty SJ, Mackinnon JL (2000) Cyclic dynamics in field vole populations and generalist predation. Journal of Animal Ecology 69:106-119

Landais C, Fenollar F, Thuny F, Raoult D (2007) From acute Q fever to endocarditis: serological

follow-up strategy. Clin Infect Dis 44:1337-1340

Lang GH (1988) Serosurvey of Coxiella burnetii infection in dairy goat herds in Ontario. A comparison of two methods of enzyme-linked immunosorbent assay. Can J Vet Res 52:37-41

Langevin SA, Bunning M, Davis B, Komar N (2001) Experimental infection of chickens as candidate sentinels for West Nile virus. Emerg Infect Dis 7:726-729

Lanszki J, Zalewski A, Horvíth G (2007) Comparison of Red Fox Vulpes Vulpes and Pine Marten Martes Martes Food Habits in a Deciduous Forest in Hungary. Wildlife Biology 13:258-271

Last JM (2001) A Dictionary of Epidemiology, 4th edn. Oxford University Press, New York

Lau SK, Li KS, Huang Y, Shek CT, Tse H, Wang M, Choi GK, Xu H, Lam CS, Guo R, Chan KH, Zheng BJ, Woo PC, Yuen KY (2010) Eco-epidemiology and complete genome comparison of SARS-related Rhinolophus bat coronavirus in China reveal bats as reservoir for acute, self-limiting infection that allows recombination events. J Virol 84:2808-2819

Leckie FM, Thirgood SJ, Redpath SM (1998) Variation in the diet of red foxes on Scotish moorland in relation to prey abundance. Ecography 21:599-604

Lecour H, Miranda M, Magro C, Rocha A, Goncalves V (1989) Human leptospirosis--a review of 50 cases. Infection 17:8-12

Leighton (2001) A serological survey of rural dogs and cats on the southwestern Canadian prairie for zoonotic pathogens., 92 edn pp 67-71

Lembo T, Hampson K, Auty H, Beesley CA, Bessell P, Packer C, Halliday J, Fyumagwa R, Hoare R, Ernest E, Mentzel C, Mlengeya T, Stamey K, Wilkins PP, Cleaveland S (2011) Serologic surveillance of anthrax in the Serengeti ecosystem, Tanzania, 1996-2009. Emerg Infect Dis 17:387-394

Lemos ER, Rozental T, Mares-Guia MA, Almeida DN, Moreira N, Silva RG, Barreira JD, Lamas CC, Favacho AR, Damasco PV (2010) Q Fever as a Cause of Fever of Unknown Origin and Thrombocytosis: First Molecular Evidence of Coxiella burnetii in Brazil. Vector Borne Zoonotic Dis 11:85-87

Leonard FC, Quinn PJ, Ellis WA, O'Farrell K (1992) Duration of urinary excretion of leptospires by cattle naturally or experimentally infected with Leptospira interrogans serovar hardjo. Vet Rec 131:435-439

Levett PN (2001) Leptospirosis. Clin Microbiol Rev 14:296-326

Levett PN, Morey RE, Galloway RL, Turner DE, Steigerwalt AG, Mayer LW (2005) Detection of pathogenic leptospires by real-time quantitative PCR. J Med Microbiol 54:45-49

Levett PN, Whittington CU (1998) Evaluation of the indirect hemagglutination assay for diagnosis of acute leptospirosis. J Clin Microbiol 36:11-14

Levy JK, Gale DW, Gale LA (2003) Evaluation of the effect of a long-term trap-neuter-return and adoption program on a free-roaming cat population. J Am Vet Med Assoc 222:42-46

Leyhausen P (1965) The communal organisation of solitary mammals. Symposia of the Zoological Society of London 14:249-263

Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, Wang H, Crameri G, Hu Z, Zhang H, Zhang J, McEachern J, Field H, Daszak P, Eaton BT, Zhang S, Wang LF (2005) Bats are natural reservoirs of SARS-like coronaviruses. Science 310:676-679

Li X, Palmer R, Trout JM, Fayer R (2003) Infectivity of microsporidia spores stored in water at environmental temperatures. J Parasitol 89:185-188

Liberg O (1980) Spacing patterns in a population of rural free-roaming domestic cats. Oikos 35:336-349

Lilenbaum W, Monteiro RV, Albuquerque CE, Ristow P, Fraguas S, Cardoso VS, Fedullo LP (2004) Leptospiral antibodies in wild felines from Rio de Janeiro Zoo, Brazil. Vet J 168:191-193

Lipman NS, Homberger FR (2003) Rodent quality assurance testing: use of sentinel animal systems. Lab Anim (NY) 32:36-43

Lippi G, Blanckaert N, Bonini P, Green S, Kitchen S, Palicka V, Vassault AJ, Plebani M (2008) Haemolysis: an overview of the leading cause of unsuitable specimens in clincal laboratories. Clin Chem Lab Med 46:764-772

Lloyd HG (1980) The Red Fox. Batsford, London

Lowder BV, Guinane CM, Ben Zakour NL, Weinert LA, Conway-Morris A, Cartwright RA, Simpson AJ, Rambaut A, Nubel U, Fitzgerald JR (2009) Recent human-to-poultry host jump, adaptation, and pandemic spread of Staphylococcus aureus. Proc Natl Acad Sci U S A 106:19545-19550

Lucherini M, Lovari S (1996) Habitat richness affects home range size in the red fox Vulpes vulpes. Behavioural Processes 36:103-105

Ludwig A, Bigras-Poulin M, Michel P, Belanger D (2010) Risk factors associated with West Nile virus mortality in American Crow populations in Southern Quebec. J Wildl Dis 46:195-208

Macdonald DW, Apps PJ (1978) The social behaviour of a group of semidependent farm cats, Felis catus: a progress report. Carnivore Genetics Newsletter 3:256-268

Macdonald DW, Bacon PJ (1982) Fox society, contact rate and rabies epizootiology. Comp Immunol Microbiol Infect Dis 5:247-256

MacLachlan NJ, Guthrie AJ (2010) Re-emergence of Bluetongue, African horse sickness, and other Orbivirus diseases. Vet Res 41:35

Madariaga MG, Rezai K, Trenholme GM, Weinstein RA (2003) Q fever: a biological weapon in your backyard. Lancet Infect Dis 3:709-721

Maddison SE, Hayes GV, Ivey MH, Tsang VC, Slemenda SB, Norman LG (1982) Fractionation of Pneumocystis carinii antigens used in an enzyme-linked immunosorbent assay for antibodies and in the production of antiserum for detecting Pneumocystis carinii antigenemia. J Clin Microbiol

15:1029-1035

Malik Peiris JS, Poon LLM, Guan Y (2009) Emergence of a novel swine-origin influenza A virus (S-OIV) H1N1 virus in humans. Journal of Clinical Virology 45:169-173

Mammal Society (2011)

Mann JS, Douglas JG, Inglis JM, Leitch AG (1986) Q fever: person to person transmission within a family. Thorax 41:974-975

Mariya R, Chaudhary P, Kumar AA, Thangapandian E, Amutha R, Srivastava SK (2006) Evaluation of a recombinant LipL41 antigen of Leptospira interrogans serovar canicola in ELISA for serodiagnosis of bovine leptospirosis. Comp Immunol Microbiol Infect Dis 29:269-277

Marrie TJ, Durant H, Williams JC, Mintz E, Waag DM (1988) Exposure to parturient cats: a risk factor for acquisition of Q fever in Maritime Canada. J Infect Dis 158:101-108

Marrie TJ, Embil J, Yates L (1993) Seroepidemiology of Coxiella burnetii among wildlife in Nova Scotia. Am J Trop Med Hyg 49:613-615

Marrie TJ, Schlech WF, III, Williams JC, Yates L (1986) Q fever pneumonia associated with exposure to wild rabbits. Lancet 1:427-429

Masuzawa T, Nakamura R, Shimizu T, Yanagihara Y (1990) Biological activities and endotoxic activities of protective antigens (PAgs) of Leptospira interrogans. Zentralbl Bakteriol 274:109-117

Mathews F, Moro D, racan R, llling M, ller N (2007) Health surveillance in wildlife reintroductions. Biological Conservation 131:338-347

Mathis A, Michel M, Kuster H, Muller C, Weber R, Deplazes P (1997) Two Encephalitozoon cuniculi strains of human origin are infectious to rabbits. Parasitology 114 (Pt 1):29-35

Mathis A, Weber R, Deplazes P (2005) Zoonotic potential of the microsporidia. Clin Microbiol Rev 18:423-445

Matthewman L, Kelly P, Hayter D, Downie S, Wray K, Bryson N, Rycroft A, Raoult D (1997) Exposure of cats in southern Africa to Coxiella burnetii, the agent of Q fever. Eur J Epidemiol 13:477-479

Matthias MA, Levett PN (2002) Leptospiral carriage by mice and mongooses on the island of Barbados. West Indian Med J 51:10-13

Maurin M, Raoult D (1999) Q fever. Clin Microbiol Rev 12:518-553

Mavin S, Hopkins PC, MacLennan A, Joss AW, Ho-Yen DO (2009) Urban and rural risks of Lyme disease in the Scottish Highlands. Scott Med J 54:24-26

McBride AJ, Athanazio DA, Reis MG, Ko AI (2005) Leptospirosis. Curr Opin Infect Dis 18:376-386

McBride AJ, Santos BL, Queiroz A, Santos AC, Hartskeerl RA, Reis MG, Ko AI (2007) Evaluation of four whole-cell Leptospira-based serological tests for diagnosis of urban leptospirosis. Clin Vaccine Immunol 14:1245-1248

McCaughey C, Montgomery WI, Twomey N, Addley M, O'Neill HJ, Coyle PV (1996) Evidence of hantavirus in wild rodents in Northern Ireland. Epidemiology and Infection 117:361-365

McCaughey C, Murray LJ, McKenna JP, Menzies FD, McCullough SJ, O'Neill HJ, Wyatt DE, Cardwell CR, Coyle PV (2010) Coxiella burnetii (Q fever) seroprevalence in cattle. Epidemiol Infect 138:21-27

McCluskey (2003) Use of sentinel herds in monitoring and surveillance systems. In: Salman MD (ed) Animal Disease Surveillance and survey systems:methods applications. Iowa State Press, Iowa, pp 119-133

McLean RG, Ubico SR, Docherty DE, Hansen WR, Sileo L, McNamara TS (2001) West Nile virus transmission and ecology in birds. Ann N Y Acad Sci 951:54-57

McMichael AJ (2004) Environmental and social influences on emerging infectious diseases: past, present and future. Philos Trans R Soc Lond B Biol Sci 359:1049-1058

McQuiston JH, Childs JE (2002) Q fever in humans and animals in the United States. Vector Borne Zoonotic Dis 2:179-191

Menkens GE, Jr., Anderson SH (1988) Estimation of Small-Mammal Population Size. Ecology 69:1952-1959

Merianos A (2007) Surveillance and response to disease emergence. Curr Top Microbiol Immunol 315:477-509

Messick JP, Smith GW, Barnes AM (1983) Serologic testing of badgers to monitor plague in southwestern Idaho. J Wildl Dis 19:1-6

Michalak K, Austin C, Diesel S, Bacon MJ, Zimmerman P, Maslow JN (1998) Mycobacterium tuberculosis infection as a zoonotic disease: transmission between humans and elephants. Emerg Infect Dis 4:283-287

Millan J, Candela MG, Lopez-Bao JV, Pereira M, Jimenez MA, Leon-Vizcaino L (2009) Leptospirosis in wild and domestic carnivores in natural areas in Andalusia, Spain. Vector Borne Zoonotic Dis 9:549-554

Miller DS, Covell DF, McLean RG, Adrian WJ, Niezgoda M, Gustafson JM, Rongstad OJ, Schultz RD, Kirk LJ, Quan TJ (2000) Serologic survey for selected infectious disease agents in swift and kit foxes from the western United States. Journal of Wildlife Diseases 36:798-805

Miller MA, Miller WA, Conrad PA, James ER, Melli AC, Leutenegger CM, Dabritz HA, Packham AE, Paradies D, Harris M, Ames J, Jessup DA, Worcester K, Grigg ME (2008) Type X Toxoplasma gondii in a wild mussel and terrestrial carnivores from coastal California: new linkages between terrestrial mammals, runoff and toxoplasmosis of sea otters. Int J Parasitol 38:1319-1328

Mills JN, Yates TL, Childs JE, Parmenter RR, Ksiazek TG, Rollin PE, Peters CJ (1995) Guidelines for working with rodents potentially infected with hantavirus. Journal of Mammalogy 76:722

Mo L, Drancourt M (2004) Monoclonal antibodies for specific detection of Encephalitozoon cuniculi.

Clin Diagn Lab Immunol 11:1060-1063

Mohn SF (1982) Experimental encephalitozoonosis in the blue fox. Clinical and serological examinations of affected pups. Acta Vet Scand 23:503-514

Molina-Lopez R, Cabezon O, Pabon M, Darwich L, Obon E, Lopez-Gatius F, Dubey JP, Almeria S (2011) High seroprevalence of Toxoplasma gondii and Neospora caninum in the Common raven (Corvus corax) in the Northeast of Spain. Res Vet Sci

Montgomery WI (1989) Population regulation in the wood mouse, Apodemus sylvaticus.I. Density dependence in the annual cycle of abundance. Journal of Animal Ecology 58:465-475

Moos A, Hackstadt T (1987) Comparative virulence of intra- and interstrain lipopolysaccharide variants of Coxiella burnetii in the guinea pig model. Infect Immun 55:1144-1150

Morens DM, Folkers GK, Fauci AS (2004) The challenge of emerging and re-emerging infectious diseases. Nature 430:242-249

Morgan ER, Tomlinson A, Hunter S, Nichols T, Roberts E, Fox MT, Taylor MA (2008) Angiostrongylus vasorum and Eucoleus aerophilus in foxes (Vulpes vulpes) in Great Britain. Vet Parasitol 154:48-57

Morimoto K, Hooper DC, Carbaugh H, Fu ZF, Koprowski H, Dietzschold B (1998) Rabies virus quasispecies: implications for pathogenesis. Proc Natl Acad Sci U S A 95:3152-3156

Morner T, Obendorf DL, Artois M, Woodford MH (2002) Surveillance and monitoring of wildlife diseases. Rev Sci Tech 21:67-76

Morris DW, MacEachern JT (2010) Active density-dependent habitat selection in a controlled population of small mammals. Ecology 91:3131-3137

Morse SS (1995) Factors in the emergence of infectious diseases. Emerg Infect Dis 1:7-15

Morse SS (2007) Global infectious disease surveillance and health intelligence. Health Aff (Millwood) 26:1069-1077

Moss SR, Turner SL, Trout RC, White PJ, Hudson PJ, Desai A, Armesto M, Forrester NL, Gould EA (2002) Molecular epidemiology of Rabbit haemorrhagic disease virus. J Gen Virol 83:2461-2467

Mostashari F, Kulldorff M, Hartman JJ, Miller JR, Kulasekera V (2003) Dead bird clusters as an early warning system for West Nile virus activity. Emerg Infect Dis 9:641-646

Muller M (1997) What are the microsporidia? Parasitol Today 13:455-456

Muller-Doblies UU, Herzog K, Tanner I, Mathis A, Deplazes P (2002) First isolation and characterisation of Encephalitozoon cuniculi from a free-ranging rat (Rattus norvegicus). Vet Parasitol 107:279-285

Munday BL (1972) A serological study of some infectious diseases of Tasmanian wildlife. J Wildl Dis 8:169-175

Murphy AM, Field PR (1970) The persistence of complement-fixing antibodies to Q-fever (Coxiella

burneti) after infection. Med J Aust 1:1148-1150

Murphy TM, Walochnik J, Hassl A, Moriarty J, Mooney J, Toolan D, Sanchez-Miguel C, O'Loughlin A, McAuliffe A (2007) Study on the prevalence of Toxoplasma gondii and Neospora caninum and molecular evidence of Encephalitozoon cuniculi and Encephalitozoon (Septata) intestinalis infections in red foxes (Vulpes vulpes) in rural Ireland. Vet Parasitol 146:227-234

Murray JK, Browne WJ, Roberts MA, Whitmarsh A, Gruffydd-Jones TJ (2010) Number and ownership profiles of cats and dogs in the UK. Veterinary Record 166:163-168

Nader IA, Martin RL (1962) The shrew as prey of the domestic cat. Journal of Mammology 43:417

National Wildlife Disease Program (2010) 2010 Plague Serosurveillance in Wildlife.

Nelson L, Clark FW (1973) Corrections for sprung traps in catch/effort calculations of trapping results. Journal of Mammalogy 54:295-298

Neumann G, Noda T, Kawaoka Y (2009) Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459:931-939

Nicholls JM, Chan RWY, Russell RJ, Air GM, Peiris JSM (2008) Evolving complexities of influenza virus and its receptors. Trends in Microbiology 16:149-157

Nichols JD, Pollock KH (1983) Estimation Methodology in Contemporary Small Mammal Capture-Recapture Studies. Journal of Mammalogy 64:253-260

Nicholson VM, Prescott JF (1993) Outer membrane proteins of three pathogenic Leptospira species. Vet Microbiol 36:123-138

Nigrovic LE, Wingerter SL (2008) Tularemia. Infect Dis Clin North Am 22:489-504, ix

Nugent G, Whitford J, Young N (2002) Use of released pigs as sentinels for Mycobacterium bovis. J Wildl Dis 38:665-677

O'Keefe JS (2002) A brief review on the laboratory diagnosis of leptospirosis. N Z Vet J 50:9-13

OIE (2008a) Leptospirosis. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, pp 251-264

OIE (2008b) Q Fever. Manual of Dioagnostic Tests and CVacciens for Terrestrial Animals. OIE, pp 292-303

OIE (2009) Terrestrial Animal Health Code.

Okewole EA (2008) Seroprevalence of antibodies to Encephalitozoon cuniculi in domestic rabbits in Nigeria. Onderstepoort J Vet Res 75:33-38

Okuda M, Sakai Y, Matsuuchi M, Oikawa T, Watanabe M, Itamoto K, Iwata H, Kano R, Hasegawa A, Onishi T, Inokuma H (2005) Enzyme-linked immunosorbent assay for the detection of canine Leptospira antibodies using recombinant OmpL1 protein. J Vet Med Sci 67:249-254

Olsen RW (1975) Length of Trapping Period in Population Studies. Journal of Mammalogy 56:696-697

Olsson GE, White N, Hjalten J, Ahlm C (2005) Habitat factors associated with bank voles (Clethrionomys glareolus) and concomitant hantavirus in northern Sweden. Vector Borne Zoonotic Dis 5:315-323

Orenstein JM, Russo P, Didier ES, Bowers C, Bunin N, Teachey DT (2005) Fatal pulmonary microsporidiosis due to encephalitozoon cuniculi following allogeneic bone marrow transplantation for acute myelogenous leukemia. Ultrastruct Pathol 29:269-276

Osterman LE, Juremalm M, Christensson D, Widgren S, Hallgren G, Agren EO, Uhlhorn H, Lindberg A, Cedersmyg M, Wahlstrom H (2011) First detection of Echinococcus multilocularis in Sweden, February to March 2011. Euro Surveill 16:

Palmer MV (2007) Tuberculosis: a reemerging disease at the interface of domestic animals and wildlife. Curr Top Microbiol Immunol 315:195-215

Palmer MV, Stoffregen WC, Carpenter JG, Stabel JR (2005) Isolation of Mycobacterium avium subsp paratuberculosis (Map) from feral cats on a dairy farm with Map-infected cattle. J Wildl Dis 41:629-635

Parker SE, Malone S, Bunte RM, Smith AL (2009) Infectious diseases in wild mice (Mus musculus) collected on and around the University of Pennsylvania (Philadelphia) Campus. Comp Med 59:424-430

Parkes JP, Heyward RP, Henning J, Motha MXJ (2004) Antibody responses to rabbit haemorrhagic disease virus in predators, scavengers, and hares in New Zealand during epidemics in sympatric rabbit populations. New Zeal Vet J 52:85-89

Parrish CR, Holmes EC, Morens DM, Park EC, Burke DS, Calisher CH, Laughlin CA, Saif LJ, Daszak P (2008) Cross-species virus transmission and the emergence of new epidemic diseases. Microbiol Mol Biol Rev 72:457-470

Patterson-Kane JC, Caplazi P, Rurangirwa F, Tramontin RR, Wolfsdorf K (2003) Encephalitozoon cuniculi placentitis and abortion in a quarterhorse mare. J Vet Diagn Invest 15:57-59

Pearce-Duvet JM (2006) The origin of human pathogens: evaluating the role of agriculture and domestic animals in the evolution of human disease. Biol Rev Camb Philos Soc 81:369-382

Pearson DE, Ruggiero LF (2003) Transect versus grid trapping arranegments for sampling small-mammal communities. Wildlife Society Bulletin 31:454-459

Pence DB, Ueckermann E (2002) Sarcoptic manage in wildlife. Rev Sci Tech 21:385-398

Peter O, Dupuis G, Bee D, Luthy R, Nicolet J, Burgdorfer W (1988) Enzyme-linked immunosorbent assay for diagnosis of chronic Q fever. J Clin Microbiol 26:1978-1982

Peter O, Dupuis G, Peacock MG, Burgdorfer W (1987) Comparison of enzyme-linked immunosorbent assay and complement fixation and indirect fluorescent-antibody tests for detection of Coxiella burnetii antibody. J Clin Microbiol 25:1063-1067

Petney TN, Andrews RH (1998) Multiparasite communities in animals and humans: frequency,

structure and pathogenic significance. Int J Parasitol 28:377-393

Petrie A, Watson P (2006) Statistics for Veterinary and Animal Science, 2nd edn. Blackwell Publsihing, Oxford

Phalen DN, Logan KS, Snowden KF (2006) Encephalitozoon hellem infection as the cause of a unilateral chronic keratoconjunctivitis in an umbrella cockatoo (Cacatua alba). Vet Ophthalmol 9:59-63

Philbey AW, Kirkland PD, Saunders GR (2005) Assessment of antibodies to rabbit haemorrhagic disease virus in fox serum as an indicator of infection in sympatric rabbit populations. Aust Vet J 83:97-100

Poonacha KB, William PD, Stamper RD (1985) Encephalitozoonosis in a parrot. J Am Vet Med Assoc 186:700-702

Pretty JN, Brett C, Gee D, Hine RE, Mason CF, Morison JIL, Raven H, Rayment MD, van der Bijl G (2000) An assessment of the total external costs of UK agriculture. Agricultural Systems 65:113-136

Puechmaille SJ, Verdeyroux P, Fuller H, Gouilh MA, Bekaert M, Teeling EC (2010) White-nose syndrome fungus (Geomyces destructans) in bat, France. Emerg Infect Dis 16:290-293

Purse BV, Brown HE, Harrup L, Mertens PP, Rogers DJ (2008) Invasion of bluetongue and other orbivirus infections into Europe: the role of biological and climatic processes. Rev Sci Tech 27:427-442

Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PP, Baylis M (2005) Climate change and the recent emergence of bluetongue in Europe. Nat Rev Microbiol 3:171-181

Qiu XF, Xu HF, Guo ZQ, Wang J, Yan J (2008) [Establishment and application of ELISAs based on rLipL32/1-LipL21-OmpL1/2 fusion antigen of Leptospira interrogans]. Zhejiang Da Xue Xue Bao Yi Xue Ban 37:592-598

Rabilloud T (2009) Detergents and chaotropes for protein solubilization before two-dimensional electrophoresis. Methods Mol Biol 528:259-267

Rabinowitz P, Gordon Z, Chudnov D, Wilcox M, Odofin L, Liu A, Dein J (2006) Animals as sentinels of bioterrorism agents. Emerg Infect Dis 12:647-652

Rabinowitz P, Scotch M, Conti L (2009) Human and animal sentinels for shared health risks. Vet Ital 45:23-24

Rabinowitz P, Wiley J, Odofin L, Wilcox M, Dein FJ (2008) Animals as sentinels of chemical terrorism agents: an evidence-based review. Clin Toxicol (Phila) 46:93-100

Rabinowitz P, Gordon Z, Holmes R, Taylor B, Wilcox M, Chudnov D, Nadkarni P, Dein F (2005) Animals as Sentinels of Human Environmental Health Hazards: An Evidence-Based Analysis. EcoHealth 2:26-37

Randolph, Chemini C, Fulanello C, Genchi C, Hails RS, Hudson PJ, Jones LD, Medley G, Norman

RA, Rizzoli AP, Smith G, Woolhouse MEJ (2002) The ecology of tick-borne infections in wildlife reservoirs. In: Hudson PJ, Rizzoli A, Grenfell BT, Heesterbeck H, Dobson AP (eds) The Ecology of Wildlife Diseases. Oxford University Press, Oxford, pp 119-138

Raoul F, Deplazes P, Nonaka N, Piarroux R, Vuitton DA, Giraudoux P (2001) Assessment of the epidemiological status of Echinococcus multilocularis in foxes in France using ELISA coprotests on fox faeces collected in the field. Int J Parasitol 31:1579-1588

Raoult D, Marrie T, Mege J (2005) Natural history and pathophysiology of Q fever. Lancet Infect Dis 5:219-226

Ready PD (2010) Leishmaniasis emergence in Europe. Euro Surveill 15:19505

Redpath CJ, Thirgood SJ, Redpath SM (1995) Evaluation of methods to estimate field vole Microtus agrestis abundance in upland habitats. Journal of Zoology 237:49-55

Reetz J (1993) [Naturally-acquired microsporidia (Encephalitozoon cuniculi) infections in hens]. Tierarztl Prax 21:429-435

Reeves NA, Helps CR, Gunn-Moore DA, Blundell C, Finnemore PL, Pearson GR, Harbour DA (1998) Natural Borna disease virus infection in cats in the United Kingdom. Veterinary Record 143:523-526

Reitstetter RE (2006) Development of species-specific PCR primer sets for the detection of Leptospira. FEMS Microbiol Lett 264:31-39

Resnick MP, Grunenwald P, Blackmar D, Hailey C, Bueno R, Murray KO (2008) Juvenile dogs as potential sentinels for West Nile virus surveillance. Zoonoses Public Health 55:443-447

Reusken C, van der Plaats R, Opsteegh M, de BA, Swart A (2011) Coxiella burnetii (Q fever) in Rattus norvegicus and Rattus rattus at livestock farms and urban locations in the Netherlands; could Rattus spp. represent reservoirs for (re)introduction? Prev Vet Med 101:124-130

Reynolds JC, Tapper SC (1995) The ecology of the red fox Vulpes vulpes in relation to small game in rural southern England. Wildlife Biology 1:105-109

Rhyan JC, Spraker TR (2010) Emergence of diseases from wildlife reservoirs. Vet Pathol 47:34-39

Ribeiro MA, Souza CC, Almeida SH (1995) Dot-ELISA for human leptospirosis employing immunodominant antigen. J Trop Med Hyg 98:452-456

Ribotta MJ, Higgins R, Gottschalk M, Lallier R (2000) Development of an indirect enzyme-linked immunosorbent assay for the detection of leptospiral antibodies in dogs. Can J Vet Res 64:32-37

Richards DT, Harris S, Lewis JW (1995) Epidemiological studies on intestinal helminth parasites of rural and urban red foxes (Vulpes vulpes) in the United Kingdom. Vet Parasitol 59:39-51

Richardson DJ, Gauthier JL (2003) A serosurvey of leptospirosis in Connecticut peridomestic wildlife. Vector Borne Zoonotic Dis 3:187-193

Richardson MD, Turner A, Warnock DW, Llewellyn PA (1983) Computer-assisted rapid enzyme-

linked immunosorbent assay (ELISA) in the serological diagnosis of aspergillosis. J Immunol Methods 56:201-207

Richter D, Schlee DB, Matuschka FR (2011) Reservoir competence of various rodents for the lyme disease Spirochete Borrelia spielmanii. Appl Environ Microbiol 77:3565-3570

Rim BM, Rim CW, Chang WH, Kakoma I (1993) Leptospirosis serology in Korean wild animals. J Wildl Dis 29:602-603

Rimmelzwaan GF, van RD, Baars M, Bestebroer TM, van AG, Fouchier RA, Osterhaus AD, Kuiken T (2006) Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. Am J Pathol 168:176-183

RIVM (National Institute for Public Health and the Environment) (2004) Zoonoses in Europe: a risk to public health. RIVM, Bilthoven, The Netherlands

Roelke-Parker ME, Munson L, Packer C, Kock R, Cleaveland S, Carpenter M, O'Brien SJ, Pospischil A, Hofmann-Lehmann R, Lutz H, Mwamengele GL, Mgasa MN, Machange GA, Summers BA, Appel MJ (1996) A canine distemper virus epidemic in Serengeti lions (Panthera leo). Nature 379:441-445

Rosenblum EB, Voyles J, Poorten TJ, Stajich JE (2010) The Deadly Chytrid Fungus: A Story of an Emerging Pathogen. PLoS Pathog 6:e1000550

Rossi P, La RG, Ludovisi A, Tamburrini A, Gomez Morales MA, Pozio E (1998) Identification of a human isolate of Encephalitozoon cuniculi type I from Italy. Int J Parasitol 28:1361-1366

Roulichova J, Andera M (2007) Simple method of age determination in red fox, Vulpes vulpes. Folia Zool 56:440-444

RSPB (2010) RSPB.

Ruiz MO, Chaves LF, Hamer GL, Sun T, Brown WM, Walker ED, Haramis L, Goldberg TL, Kitron UD (2010) Local impact of temperature and precipitation on West Nile virus infection in Culex species mosquitoes in northeast Illinois, USA. Parasit Vectors 3:19

Ruiz-Fons F, Rodriguez O, Torina A, Naranjo V, Gortazar C, de la FJ (2008) Prevalence of Coxiella burnetti infection in wild and farmed ungulates. Vet Microbiol 126:282-286

Saitoh T, Bjornstad ON, Stenseth NC (1999) Density dependence in voles and mice:A comparative study. Ecology 80:638-650

Sak B, Kvac M, Kvetonova D, Albrecht T, Pialek J (2011) The first report on natural Enterocytozoon bieneusi and Encephalitozoon spp. infections in wild East-European House Mice (Mus musculus musculus) and West-European House Mice (M. m. domesticus) in a hybrid zone across the Czech Republic-Germany border. Vet Parasitol 178:246-250

Salkeld DJ, Stapp P (2006) Seroprevalence rates and transmission of plague (Yersinia pestis) in mammalian carnivores. Vector Borne Zoonotic Dis 6:231-239

Salman (2003) Surveillance and monitoring systems for animal health programs and disease surveys. In: Salman MD (ed) Animal Disease Surveillance and survey systems:methods applications. Iowa State Press, Iowa, pp 3-13

Sangster C, Bergeson D, Lutze-Wallace C, Crichton V, Wobeser G (2007) Feasibility of using coyotes (Canis latrans) as sentinels for bovine mycobacteriosis (Mycobacterium bovis) infection in wild cervids in and around Riding Mountain National Park, Manitoba, Canada. J Wildl Dis 43:432-438

Sansonetti P (2006) [How to define the species barrier to pathogen transmission?]. Bull Acad Natl Med 190:611-622

Santaniello A, Dipineto L, Rinaldi L, Menna LF, Cringoli G, Fioretti A (2009) Serological survey of Encephalitozoon cuniculi in farm rabbits in Italy. Res Vet Sci 87:67-69

Schwartz DA, Bryan RT, Weber R, Visvesvara GS (1994) Microsporidiosis in HIV positive patients: current methods for diagnosis using biopsy, cytologic, ultrastructural, immunological, and tissue culture techniques. Folia Parasitol (Praha) 41:101-109

Scotch M, Odofin L, Rabinowitz P (2009) Linkages between animal and human health sentinel data. BMC Vet Res 5:15

Scott AC, Wells GA, Stack MJ, White H, Dawson M (1990) Bovine spongiform encephalopathy: detection and quantitation of fibrils, fibril protein (PrP) and vacuolation in brain. Vet Microbiol 23:295-304

Scottish Wildcat Association (2011)

Sepulveda MA, Munoz-Zanzi C, Rosenfeld C, Jara R, Pelican KM, Hill D (2011) Toxoplasma gondii in feral American minks at the Maullin river, Chile. Vet Parasitol 175:60-65

Shannon JG, Heinzen RA (2009) Adaptive immunity to the obligate intracellular pathogen Coxiella burnetii. Immunol Res 43:138-148

Shaw SE, Hillman T, Wray J (2008) Unusual case of canine leishmaniosis in the UK. Vet Rec 163:283

Shaw SE, Langton DA, Hillman TJ (2009) Canine leishmaniosis in the United Kingdom: a zoonotic disease waiting for a vector? Vet Parasitol 163:281-285

Shimizu T, Matsusaka E, Nagakura N, Takayanagi K, Masuzawa T, Iwamoto Y, Morita T, Mifuchi I, Yanagihara Y (1987) Chemical properties of lipopolysaccharide-like substance (LLS) extracted from Leptospira interrogans serovar canicola strain Moulton. Microbiol Immunol 31:717-725

Shingadia D, Novelli V (2008) The tuberculin skin test: a hundred, not out? Arch Dis Child 93:189-190

Shope RE, Hurst EW (1933) Infectious papillomatosis of rabbits : with a note on the histopathology. J Exp Med 58:607-624

Shophet R (1979) A serological survey of leptospirosis in cats. N Z Vet J 27:236, 245-236, 246

Shophet R, Marshall RB (1980) An experimentally induced predator chain transmission of Leptospira ballum from mice to cats. Br Vet J 136:265-270

Slaba K, Skultety L, Toman R (2005) Efficiency of various serological techniques for diagnosing Coxiella burnetii infection. Acta Virol 49:123-127

Slavica A, Konjevic D, Huber D, Milas Z, Turk N, Sindicic M, Severin K, Dezdek D, Masek T (2010) Serologic evidence of Leptospira spp. serovars in brown bears (Ursus arctos) from Croatia. J Wildl Dis 46:251-256

Sleeman (2011) Use of wildlife rehabilitation centers as monitors of ecosystem health. In: Fowler ME, Miller RE (eds) Zoo and Wild Animal Medicine:Current Therapy, 6 edn. Saunders, St. Louis, pp 97-104

Smith AL, Singleton GR, Hansen GM, Shellam G (1993) A serologic survey for viruses and Mycoplasma pulmonis among wild house mice (Mus domesticus) in southeastern Australia. J Wildl Dis 29:219-229

Smith GC, Gangadharan B, Taylor Z, Laurenson MK, Bradshaw H, Hide G, Hughes JM, Dinkel A, Romig T, Craig PS (2003) Prevalence of zoonotic important parasites in the red fox (Vulpes vulpes) in Great Britain. Vet Parasitol 118:133-142

Smits HL, Eapen CK, Sugathan S, Kuriakose M, Gasem MH, Yersin C, Sasaki D, Pujianto B, Vestering M, Abdoel TH, Gussenhoven GC (2001) Lateral-flow assay for rapid serodiagnosis of human leptospirosis. Clin Diagn Lab Immunol 8:166-169

Smits HL, Hartskeerl RA, Terpstra WJ (2000) International multi-centre evaluation of a dipstick assay for human leptospirosis. Trop Med Int Health 5:124-128

Smythe LD, Smith IL, Smith GA, Dohnt MF, Symonds ML, Barnett LJ, McKay DB (2002) A quantitative PCR (TaqMan) assay for pathogenic Leptospira spp. BMC Infect Dis 2:13

Snowden K, Logan K, Didier ES (1999) Encephalitozoon cuniculi strain III is a cause of encephalitozoonosis in both humans and dogs. J Infect Dis 180:2086-2088

Song HD, Tu CC, Zhang GW, Wang SY, Zheng K, Lei LC, Chen QX, Gao YW, Zhou HQ, Xiang H, Zheng HJ, Chern SW, Cheng F, Pan CM, Xuan H, Chen SJ, Luo HM, Zhou DH, Liu YF, He JF, Qin PZ, Li LH, Ren YQ, Liang WJ, Yu YD, Anderson L, Wang M, Xu RH, Wu XW, Zheng HY, Chen JD, Liang G, Gao Y, Liao M, Fang L, Jiang LY, Li H, Chen F, Di B, He LJ, Lin JY, Tong S, Kong X, Du L, Hao P, Tang H, Bernini A, Yu XJ, Spiga O, Guo ZM, Pan HY, He WZ, Manuguerra JC, Fontanet A, Danchin A, Niccolai N, Li YX, Wu CI, Zhao GP (2005) Cross-host evolution of severe acute respiratory syndrome coronavirus in palm civet and human. Proc Natl Acad Sci U S A 102:2430-2435

Soulsbury CD, Iossa G, Baker PJ, Harris S (2008) Environmental variation at the onset of independent foraging affects full-grown body mass in the red fox. Proc Biol Sci 275:2411-2418

Soverow JE, Wellenius GA, Fisman DN, Mittleman MA (2009) Infectious disease in a warming world: how weather influenced West Nile virus in the United States (2001-2005). Environ Health Perspect 117:1049-1052

Stokes JE, Kaneene JB, Schall WD, Kruger JM, Miller R, Kaiser L, Bolin CA (2007) Prevalence of serum antibodies against six Leptospira serovars in healthy dogs. J Am Vet Med Assoc 230:1657-1664

Suarez DL (2005) Overview of avian influenza DIVA test strategies. Biologicals 33:221-226

Sullivan EG (1956) Age Determination of foxes by X-ray of forefeet. Journal of Wildlife Management 20:210-212

Tabor R (1983) The wildlife of the domestic cat. Arrow Books, London

Takimoto K, Taharaguchi M, Morikawa S, Ike F, Yamada YK (2008) Detection of the antibody to lymphocytic choriomeningitis virus in sera of laboratory rodents infected with viruses of laboratory and newly isolated strains by ELISA using purified recombinant nucleoprotein. Exp Anim 57:357-365

Tanaka R (1960) Evidence against reliability of the trap-night index as a relative measure of population in small mammals. Japanese Journal of Ecology 10:102-106

Tangkanakul W, Smits HL, Jatanasen S, Ashford DA (2005) Leptospirosis: an emerging health problem in Thailand. Southeast Asian J Trop Med Public Health 36:281-288

Taylor LH, Latham SM, Woolhouse ME (2001) Risk factors for human disease emergence. Philos Trans R Soc Lond B Biol Sci 356:983-989

Teachey DT, Russo P, Orenstein JM, Didier ES, Bowers C, Bunin N (2004) Pulmonary infection with microsporidia after allogeneic bone marrow transplantation. Bone Marrow Transplant 33:299-302

Telfer S, Bennett M, Carslake D, Helyar S, Begon M (2007) The dynamics of murid gammaherpesvirus 4 within wild, sympatric populations of bank voles and wood mice. J Wildl Dis 43:32-39

Telfer S, Birtles R, Bennett M, Lambin X, Paterson S, Begon M (2008) Parasite interactions in natural populations: insights from longitudinal data. Parasitology 135:767-781

Telfer S, Lambin X, Birtles R, Beldomenico P, Burthe S, Paterson S, Begon M (2010) Species interactions in a parasite community drive infection risk in a wildlife population. Science 330:243-246

Tew TE, Todd IA, Macdonald DW (1994) The effects of trap spacing on population estimatation of small mammals. Journal of Zoology 233:340-344

The Scottish Government (2007) Report on urban fox populations in Scottish towns and cities.

Thiermann AB (1981) The Norway rat as a selective chronic carrier of Leptospira icterohaemorrhagiae. J Wildl Dis 17:39-43

Thiermann AB, Garrett LA (1983) Enzyme-linked immunosorbent assay for the detection of antibodies to Leptospira interrogans serovars hardjo and pomona in cattle. Am J Vet Res 44:884-887

Thomas C, Finn M, Twigg L, Deplazes P, Thompson RC (1997) Microsporidia (Encephalitozoon cuniculi) in wild rabbits in Australia. Aust Vet J 75:808-810

To H, Sakai R, Shirota K, Kano C, Abe S, Sugimoto T, Takehara K, Morita C, Takashima I, Maruyama T, Yamaguchi T, Fukushi H, Hirai K (1998) Coxiellosis in domestic and wild birds from Japan. J Wildl Dis 34:310-316

Trewhella WJ, Harris S, McAllister FE (1988) Dispersal Distance, Home-Range Size and Population Density in the Red Fox (Vulpes vulpes): A Quantitative Analysis. Journal of Applied Ecology 25:423-434

Trout RC, Chasey D, Sharp G (1997) Seroepidemiology of rabbit haemmorhagic disease (RHD) in wild rabbits (Oryctolagus cuniculus). Journal of Zoology 243:846-853

Tryland M, Handeland K, Bratberg AM, Solbakk IT, Oksanen A (2006) Persistence of antibodies in blood and body fluids in decaying fox carcasses, as exemplified by antibodies against Microsporum canis. Acta Vet Scand 48:10

Turner DC, Mertens C (1986) Home Range Size, Overlap and Exploitation in Domestic Farm Cats (Felis catus). Behaviour 99:22-45

Twigg GI, Cox PJ (1976) The distribution of leptospires in the kidney tubules of some British wild mammals. J Wildl Dis 12:318-321

Twigg GI, Cuerden CM, Hughes DM, Medhurst P (1969) The leptospirosis reservoir in British wild mammals. Vet Rec 84:424-426

Tyler JW, Cullor JS (1989) Titers, tests, and truisms: rational interpretation of diagnostic serologic testing. J Am Vet Med Assoc 194:1550-1558

UNAIDS (2008) 2008 Report on the global AIDS epidemic.

van der Schalie WH, Gardner HS, Jr., Bantle JA, De Rosa CT, Finch RA, Reif JS, Reuter RH, Backer LC, Burger J, Folmar LC, Stokes WS (1999) Animals as sentinels of human health hazards of environmental chemicals. Environ Health Perspect 107:309-315

van der HW, Dijkstra F, Schimmer B, Schneeberger PM, Vellema P, Wijkmans C, ter SR, Hackert V, van DY (2010) Q fever in the Netherlands: an update on the epidemiology and control measures. Euro Surveill 15:

Van Heerden J., Bainbridge N, Burroughs RE, Kriek NP (1989) Distemper-like disease and encephalitozoonosis in wild dogs (Lycaon pictus). J Wildl Dis 25:70-75

van Rensburg IB, du Plessis JL (1971) Nosematosis in a cat:a case report. Journal of the South African Veterinary Association 42:327-331

van GT, Biderre C, Delbac F, Wentink-Bonnema E, Peek R, Vivares CP (2004) Serodiagnostic studies in an immunocompetent individual infected with Encephalitozoon cuniculi. J Infect Dis 189:2243-2249

van MP, Baumgartner W, Eskens U, Hanichen T (1993) Immunocytochemical demonstration of Coxiella burnetii antigen in the fetal placenta of naturally infected sheep and cattle. J Comp Pathol 109:295-301

van RB, Langelaar M, van der GJ, Roest HJ, Grone A (2010) [Q fever in wild animals in Europe, attention to hunters]. Tijdschr Diergeneeskd 135:420-422

Vanasco NB, Lottersberger J, Sequeira MD, Tarabla H (2001) Development and validation of an ELISA for the detection of leptospire-specific antibodies in rodents. Vet Microbiol 82:321-330

VerCauteren KC, Atwood TC, DeLiberto TJ, Smith HJ, Stevenson JS, Thomsen BV, Gidlewski T, Payeur J (2008) Surveillance of coyotes to detect bovine tuberculosis, Michigan. Emerg Infect Dis 14:1862-1869

Vijayachari P, Sugunan AP, Shriram AN (2008) Leptospirosis: an emerging global public health problem. J Biosci 33:557-569

Voigt DR, Macdonald DW (1984) Variation in the spatial and social behaviour of the red fox Vulpes vulpes. Acta Zool Fenn , 171:261-265

Waag D, Chulay J, Marrie T, England M, Williams J (1995) Validation of an enzyme immunoassay for serodiagnosis of acute Q fever. Eur J Clin Microbiol Infect Dis 14:421-427

Waag DM (2007) Coxiella burnetii: host and bacterial responses to infection. Vaccine 25:7288-7295

Wahlstrom H, Isomursu M, Hallgren G, Christensson D, Cedersmyg M, Wallensten A, Hjertqvist M, Davidson RK, Uhlhorn H, Hopp P (2011) Combining information from surveys of several species to estimate the probability of freedom from Echinococcus multilocularis in Sweden, Finland and mainland Norway. Acta Vet Scand 53:9

Walker SF, Bosch J, Gomez V, Garner TW, Cunningham AA, Schmeller DS, Ninyerola M, Henk DA, Ginestet C, Arthur CP, Fisher MC (2010) Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. Ecol Lett 13:372-382

Wallensten A, Moore P, Webster H, Johnson C, van der BG, Pritchard G, Ellis-Iversen J, Oliver I (2010) Q fever outbreak in Cheltenham, United Kingdom, in 2007 and the use of dispersion modelling to investigate the possibility of airborne spread. Euro Surveill 15:pii 19521

Wang H, Nagy JD, Gilg O, Kuang Y (2009) The roles of predator maturation delay and functional response in determining the periodicity of predator-prey cycles. Math Biosci 221:1-10

Ward MP, Glickman LT, Guptill LE (2002) Prevalence of and risk factors for leptospirosis among dogs in the United States and Canada: 677 cases (1970-1998). J Am Vet Med Assoc 220:53-58

Ward MR, Stallknecht DE, Willis J, Conroy MJ, Davidson WR (2006) Wild bird mortality and West Nile virus surveillance: biases associated with detection, reporting, and carcass persistence. J Wildl Dis 42:92-106

Warner RE (1985) Demography and movements of free-ranging domestic cats in rural Illinois. Journal of Wildlife Management 49:340-346

Washington J.A (1996) Principles of Diagnosis. In: Baron S (ed) Medical Microbiology, 4th edn. University of Texas Medical Branch, Galveston, TX,

Wasson K, Peper RL (2000) Mammalian microsporidiosis. Vet Pathol 37:113-128

Webbon CC, Baker PJ, Harris S (2004) Faecal density counts for monitoring changes in red fox numbers in rural Britain . Journal of Applied Ecology 41:768-779

Weber R, Deplazes P, Flepp M, Mathis A, Baumann R, Sauer B, Kuster H, Luthy R (1997) Cerebral microsporidiosis due to Encephalitozoon cuniculi in a patient with human immunodeficiency virus infection. N Engl J Med 336:474-478

Webster JP, Ellis WA, Macdonald DW (1995a) Prevalence of Leptospira spp. in wild brown rats (Rattus norvegicus) on UK farms. Epidemiol Infect 114:195-201

Webster JP, Lloyd G, Macdonald DW (1995b) Q fever (Coxiella burnetii) reservoir in wild brown rat (Rattus norvegicus) populations in the UK. Parasitology 110 (Pt 1):31-35

Webster JP, Macdonald DW (1995) Parasites of wild brown rats (Rattus norvegicus) on UK farms. Parasitology 111 (Pt 3):247-255

Weihong JI, Veitch CR, Craig JL (1999) An evaluation of the efficiency of rodent trapping methods:the effect of trap arrangement, cover type and bait. New Zealand Journal of Ecology 23:45-51

Welburn S (2011) One Health: the 21st century challenge. Vet Rec 168:614-615

Weldon C, du Preez LH, Hyatt AD, Muller R, Spears R (2004) Origin of the amphibian chytrid fungus. Emerg Infect Dis 10:2100-2105

Whitney EA, Massung RF, Candee AJ, Ailes EC, Myers LM, Patterson NE, Berkelman RL (2009) Seroepidemiologic and occupational risk survey for Coxiella burnetii antibodies among US veterinarians. Clin Infect Dis 48:550-557

WHO (2003) Summary of probable SARS cases with onset of illness from 1 november 2002 to 31 July 2003.

Wild TF (2009) Henipaviruses: a new family of emerging Paramyxoviruses. Pathol Biol (Paris) 57:188-196

Williams ES, Thorne ET, Appel MJ, Belitsky DW (1988) Canine distemper in black-footed ferrets (Mustela nigripes) from Wyoming. J Wildl Dis 24:385-398

Williams ES, Yuill T, Artois M, Fischer J, Haigh SA (2002) Emerging infectious diseases in wildlife. Rev Sci Tech 21:139-157

Wilson JM (1979) Encephalitozoon cuniculi in wild European rabbits and a fox. Res Vet Sci 26:114

Wobeser G, Campbell GD, Dallaire A, McBurney S (2009) Tularemia, plague, yersiniosis, and Tyzzer's disease in wild rodents and lagomorphs in Canada: a review. Can Vet J 50:1251-1256

Woldehiwet Z (2004) Q fever (coxiellosis): epidemiology and pathogenesis. Res Vet Sci 77:93-100

Wolfe ND, Dunavan CP, Diamond J (2007) Origins of major human infectious diseases. Nature 447:279-283

Wolfe ND, Switzer WM, Carr JK, Bhullar VB, Shanmugam V, Tamoufe U, Prosser AT, Torimiro JN, Wright A, Mpoudi-Ngole E, McCutchan FE, Birx DL, Folks TM, Burke DS, Heneine W (2004) Naturally acquired simian retrovirus infections in central African hunters. Lancet 363:932-937

Woods M, McDonald RA, Harris S (2003) Predation of wildlife by domestic cats Felis catus in Great Britain. Mammal Review 33:174-188

Woolhouse M, Gaunt E (2007) Ecological origins of novel human pathogens. Crit Rev Microbiol 33:231-242

Woolhouse ME (2002) Population biology of emerging and re-emerging pathogens. Trends Microbiol 10:S3-S7

Woolhouse ME, Gowtage-Sequeria S (2005) Host range and emerging and reemerging pathogens. Emerg Infect Dis 11:1842-1847

Woolhouse ME, Haydon DT, Antia R (2005) Emerging pathogens: the epidemiology and evolution of species jumps. Trends Ecol Evol 20:238-244

Woolhouse ME, Taylor LH, Haydon DT (2001) Population biology of multihost pathogens. Science 292:1109-1112

World Health Organisation (1959) Zoonoses:second report of the joint WHO/FAO expert committee., Technical Report Series No 169 edn. World Health Organisation, Geneva,

World Health Organisation (1999) Leptospirosis worldwide. Weekly Epidemiological Record 74:237-242

World Health Organisation (2001) Protocol for the evaluation of epidemiological surveillance systems.

World Health Organisation (2003) Human Leptospirosis: Guidance for diagnosis, surveillance and control. WHO,

World Health Organisation (2009) World Malaria Report.

World Organisation for Animal Health (2010) World Organisation for Animal Health. Terrestrial Animal Health Code 2009.

Worley MB (1983) Molecular biology and infectious diseases:present and future trends in diagnosis. Journal of Zoo and Wildlife Medicine 24:336-345

Xavier T, Ganesan TS, Menon KN (2010) A simple and efficient method for processing of cell lysates for two-dimensional gel electrophoresis. Electrophoresis 31:2429-2435

Xu Y, Weiss LM (2005) The microsporidian polar tube: a highly specialised invasion organelle. Int J Parasitol 35:941-953

Xu ZY, Tang YW, Kan LY, Tsai TF (1987) Cats--source of protection or infection? A case-control

study of hemorrhagic fever with renal syndrome. Am J Epidemiol 126:942-948

Yabsley MJ, Jordan CN, Mitchell SM, Norton TM, Lindsay DS (2007) Seroprevalence of Toxoplasma gondii, Sarcocystis neurona, and Encephalitozoon cuniculi in three species of lemurs from St. Catherines Island, GA, USA. Veterinary Parasitology 144:28-32

Yan KT, Ellis WA, Mackie DP, Taylor MJ, McDowell SW, Montgomery JM (1999) Development of an ELISA to detect antibodies to a protective lipopolysaccharide fraction of Leptospira borgpetersenii serovar hardjo in cattle. Vet Microbiol 69:173-187

Yaremych SA, Warner RE, Mankin PC, Brawn JD, Raim A, Novak R (2004) West Nile virus and high death rate in American crows. Emerg Infect Dis 10:709-711

Zachariah A (2005) Evidence for Leptospira interrogans infection in phocids in the UK. University of London,

Zarnke RL (1983) Serologic survey for selected microbial pathogens in Alaskan wildlife. J Wildl Dis 19:324-329

Zell R, Krumbholz A, Wutzler P (2008) Impact of global warming on viral diseases: what is the evidence? Curr Opin Biotechnol 19:652-660

Zinsstag J, Schelling E, Waltner-Toews D, Tanner M (2010) From "one medicine" to "one health" and systemic approaches to health and well-being. Prev Vet Med 101:148-156

Zippin C (1958) The removal method of population estimation. Journal of Wildlife Management 22:82-90

APPENDIX 1

JOURNAL OF THE ROYAL SOCIETY Interface

J. R. Soc. Interface (2007) **4**, 973–984
doi:10.1098/rsif.2007.0237
Published online 15 May 2007

A framework for evaluating animals as sentinels for infectious disease surveillance

Jo E. B. Halliday*, Anna L. Meredith, Darryn L. Knobel, Darren J. Shaw, Barend M. de C. Bronsvoort and Sarah Cleaveland

Wildlife and Emerging Diseases Section, R(D)SVS, University of Edinburgh, Easter Bush Veterinary Centre, Roslin, Midlothian EH25 9RG, UK

The dynamics of infectious diseases are highly variable. Host ranges, host responses to pathogens and the relationships between hosts are heterogeneous. Here, we argue that the use of animal sentinels has the potential to use this variation and enable the exploitation of a wide range of pathogen hosts for surveillance purposes. Animal sentinels may be used to address many surveillance questions, but they may currently be underused as a surveillance tool and there is a need for improved interdisciplinary collaboration and communication in order to fully explore the potential of animal sentinels. In different contexts, different animal hosts will themselves vary in their capacity to provide useful information. We describe a conceptual framework within which the characteristics of different host populations and their potential value as sentinels can be evaluated in a broad range of settings.

Keywords: sentinel; surveillance; infectious diseases; epidemiology

1. INTRODUCTION

The dynamics of infectious disease systems are inherently variable. The outcome of any infection depends on multiple factors relating to pathogen characteristics, host susceptibility, infecting dose and routes of transmission, all of which can vary widely for any particular infectious organism. Many of the major diseases of medical, veterinary and conservation importance (such as highly pathogenic avian influenza (HPAI), foot-and-mouth disease, bluetongue and rabies) are caused by pathogens with wide host ranges (Woolhouse & Gowtage-Sequeria 2005), which introduces further complexity.

While the complex epidemiology of multi-host pathogens presents considerable challenges for understanding infection dynamics and implementing disease control, heterogeneities in host range and infection outcome also provide opportunities for disease surveillance. In this paper, we develop a conceptual framework that can be applied to examine those characteristics of host populations that influence their potential value as sentinels for disease surveillance in different ecological and epidemiological settings.

Surveillance is defined by the World Health Organization as 'the ongoing systematic collection, collation, analysis and interpretation of data and the dissemination of information to those who need to know in order for action to be taken' (World Health Organization 2001). The aim of disease surveillance is to identify changes in the infection and/or health status of animal and human populations and is essential to provide rigorous evidence of the absence of disease or to determine the prevalence of a pathogen when present (Salman 2003). A critical element of surveillance is that an identified response is made on the basis of the surveillance data generated to allow appropriate action to be taken. Sentinel surveillance is one form of surveillance in which activities focus on specific subpopulations to enhance detection of disease and/or improve the cost-effectiveness of surveillance (McCluskey 2003). The aim of the sentinel surveillance is to obtain timely information in a relatively inexpensive manner rather than to derive precise estimates of prevalence or incidence in the general population (Centers for Disease Control and Prevention 2002). It has long been recognized that animal populations have the potential to act as sentinels for environmental health hazards (CAMEH 1991), but, given the importance of domestic and wild animal hosts in emerging human diseases, it is clear that surveillance in animals is also critical for understanding and managing emerging disease threats (Kuiken *et al.* 2005; Woolhouse & Gowtage-Sequeria 2005; Kahn 2006). Animal sentinels almost certainly represent an important but underused surveillance tool (Rabinowitz *et al.* 2005) that may be capable of accommodating and capitalizing on the variability that exists in infectious disease processes.

Animal sentinels may potentially be used to address a range of surveillance questions including (i) detection of a pathogen in a new area, (ii) detection of changes in the prevalence or incidence of a pathogen or disease over time, (iii) determining the rates and direction of

*Author for correspondence (s0454869@sms.ed.ac.uk).

One contribution of 20 to a Theme Issue 'Cross-scale influences on epidemiological dynamics: from genes to ecosystems'.

Received 7 February 2007
Accepted 11 April 2007

Table 1. Summary of applications of animal sentinels for environmental and infectious hazards.

type of sentinel	example	reference
individual animal	coal miner's canary used to detect the presence of carbon monoxide	Burrell & Seibert (1916); Schwabe (1984)
herd/population	sentinel cattle herds and chicken flocks used to monitor the distribution of arboviruses and their vectors in Australia and the USA	National Arbovirus Monitoring Program (2003–2004); Loftin *et al.* (2006)
same species	unvaccinated chickens placed within vaccinated flock to detect HPAI	Suarez (2005)
different, more susceptible species	feral pigs released into New Zealand to detect the presence of bovine TB—more susceptible than possums; coal miner's canary (as above)	Nugent *et al.* (2002)
sentinel application	**example**	**reference**
deliberately placed (experimental)	standard laboratory mice sentinel programmes using outbred mice, sacrificed and tested to detect presence of a panel of rodent pathogens in the core experimental or breeding colony	Institute of Laboratory Animal Resources (US). Committee on Infectious Diseases of Mice and Rats (1991)
	use of sentinel chickens to evaluate the effectiveness of cleaning and disinfection procedures for eradication of Newcastle disease	McCluskey *et al.* (2006)
in natural habitat (observational)	wildlife as detectors of DDT and PCB toxicity	CAMEH (1991)
	evaluation of white-tailed deer as natural sentinels for *Anaplasma phagocytophilum*, the cause of human granulocytic anaplasmosis	Dugan *et al.* (2006)
	mesothelioma in pet dogs associated with exposure of their owners to asbestos	Glickman *et al.* (1983)
sentinel unit	equine premises used to investigate presence of vesicular stomatitis in Colorado	McCluskey *et al.* (2002)

pathogen spread, (iv) testing specific hypotheses about the ecology of a pathogen, and (v) evaluating the efficacy of potential disease control interventions (McCluskey 2003). The appropriate use of animal sentinels can facilitate the early detection and identification of outbreaks that is of critical importance for the success of control and prevention efforts (Chomel 2003; Kahn 2006) and reducing the magnitude of subsequent outbreaks (Ferguson *et al.* 2005). However, the potential of animal sentinel surveillance can only be realized if the information provided from animal populations is acted upon. For example, in an Ebola outbreak in central Africa, few preventive health measures were taken despite warnings of an imminent human outbreak being provided from monitoring of Ebola deaths in primate sentinels (Rouquet *et al.* 2005).

The term 'sentinel' is widely used in both epidemiological and veterinary clinical literature and is implicitly understood but rarely defined. While all uses invoke the common concept of standing guard or keeping watch, existing definitions tend to be context-specific. The classic example of an animal sentinel is that of the coal miner's canary. In this case, an individual animal of a different species is deliberately selected and placed in a situation where it can provide evidence of increased risk to the human population on the basis of its greater sensitivity and obvious observable response to the presence of carbon monoxide. Since the mid-twentieth century, it has been recognized that animals can act as important sentinels for a wide range of environmental health hazards (CAMEH 1991). For example, domestic dogs and the tumours they develop may facilitate identification of environmental carcinogens that affect humans (Thrusfield 2005). Sentinels can vary from individual animals to herds or larger populations, from animals of the same species to different, more susceptible, more expendable or more accessible species, and from animals deliberately placed or introduced to those already existing in a particular location. The sentinel concept can also refer to a physical location, such as a farm, abattoir, veterinary practice or laboratory (the 'sentinel unit') which is selected to monitor a particular disease (table 1). Throughout this paper, we use 'animal sentinels' as an umbrella term for the topic in general and 'sentinel population' to refer to the unit of observation in a particular case.

Despite the apparent potential for animal sentinels to inform decisions about risk to both human and animal populations, animal sentinels appear underutilized, particularly in the context of infectious disease surveillance (Rabinowitz *et al.* 2005), and their value has been discussed primarily in the context of environmental health (CAMEH 1991). A basic lack of integration between disciplines, most noticeably between human and veterinary medicine and also between different branches within these fields, is likely to have contributed to this underuse of animal sentinels (Rabinowitz *et al.* 2005). There are currently no standard criteria which are applied for the evaluation of animal sentinels, limiting the ease with which data can be transferred between disciplines (Rabinowitz *et al.* 2005). The existing infectious disease literature regarding animal sentinels consists largely of descriptive studies that have generated hypotheses regarding animal sentinel use (Rabinowitz *et al.* 2005), but as yet includes few studies that were

Box 1. West Nile virus surveillance in North America: animal sentinel case study.

West Nile virus (WNV), an arbovirus of the genus *Flaviviridae*, is maintained in a mosquito–bird–mosquito cycle primarily involving *Culex* sp. mosquitoes (Campbell *et al.* 2002). Humans and other mammal species are incidental dead-end hosts. The majority of human infections with WNV are asymptomatic or result in transient febrile illness but in a small proportion of cases, meningoencephalitis can occur (Mostashari *et al.* 2001). The geographical range of WNV has historically included Africa, Europe, Asia and Australia (Campbell *et al.* 2002). In 1999, the first North American cases of WNV were reported in New York and since then the virus has spread across the continental United States and into Canada, Latin America and the Caribbean (Hayes & Gubler 2006). The surveillance of WNV in North America has included investigation of the utility of different animal sentinels. Some of the findings of these studies are described below with reference to the sentinel framework.

Sentinel response to pathogen

A number of North American bird species including corvids, house sparrows, house finches and grackles are competent reservoirs for mosquito infection with WNV (Komar *et al.* 2003). Among these potential sentinel species, corvids and specifically American crows (*Corvus brachyrhynchos*) are particularly susceptible to infection with WNV and have a high mortality rate (McLean *et al.* 2001; Komar *et al.* 2003; Yaremych *et al.* 2004). In 2000, it was established that dead crow reports preceded both the confirmation of viral activity (through laboratory analysis) and the onset of human cases by several months (Eidson *et al.* 2001*b*). Subsequent spatial analyses using data collected in New York have identified a positive association between the risk of human disease caused by WNV and elevated local dead crow reports in the previous one to two weeks (Mostashari *et al.* 2003; Eidson *et al.* 2005; Johnson *et al.* 2006). The thorough characterization of this temporal association ensures that the observation of crow deaths can be acted upon immediately without the need for time-consuming laboratory analyses. The observation of clusters of high crow mortality can therefore be used to predict human risk early enough to implement targeted mosquito control and personal protection warnings (Mostashari *et al.* 2003; Eidson *et al.* 2005; Johnson *et al.* 2006).

Relationship between sentinel and target populations

Domestic dogs have also been evaluated as sentinels of WNV presence (Komar *et al.* 2001; Kile *et al.* 2005). This sentinel choice is informed by the particular relationship that domestic dogs have with humans, which means that they are well suited to act as indicators of the infectious disease risks that their owners are likely to encounter. North American domestic dogs consistently show higher seroprevalence of anti-WNV antibodies than humans (Komar *et al.* 2001; Kile *et al.* 2005) and one analysis revealed that outdoor dogs were nearly 19 times more likely to have seroconverted to WNV than indoor-only pet dogs (Kile *et al.* 2005). The pattern of human exposure to the arthropod vectors of WNV is likely to be more similar to that of indoor-only dogs, but within the context of broad spatial association with humans; this divergence from the human niche means that outdoor-only dogs are more sensitive sentinels of WNV presence and human risk than indoor-only dogs (Kile *et al.* 2005).

Transmission route

The role played by different mosquito species (predominantly of the genus *Culex*) in the transmission of WNV between birds and to humans is apparently quite variable (Kilpatrick *et al.* 2005; Molaei *et al.* 2006). At one study site in Maryland and Washington DC, over 90% of all *Culex* mosquitoes identified were of the species *Culex pipiens* (Kilpatrick *et al.* 2006). At this site, the rise in human WNV cases that occurs in late summer and early autumn is apparently caused by a marked shift in the feeding preferences of this vector species from birds to humans (Kilpatrick *et al.* 2006) that is associated with the dispersal of a preferred host, the America robin (*Turdus migratorius*). This temporal variation in vector feeding preferences means that the transmission of WNV to bird hosts (including corvids) occurs earlier in the season than transmission to humans and explains the capacity for bird die-offs to provide an early warning of human risk. A similar shift in feeding patterns associated with a rise in human cases is also seen in *Culex tarsalis* mosquitoes in Colorado and California (Kilpatrick *et al.* 2006).

Detectability

Although the pathogenicity of WNV to birds including crows has been demonstrated within the historical geographical range of WNV (Work *et al.* 1955), bird die-offs are not typically associated with human WNV outbreaks within this historical geographical range and the very high mortality seen in American corvid populations is apparently unusual (Eidson *et al.* 2001*a*). Clearly, this difference may limit the application of corvids as useful sentinels of WNV to contexts within the Americas. Even within North America, there is variation in the suitability of corvids to act as a sentinel for WNV activity according to the density of human populations. A study using decoy crows revealed that both detection and reporting rates were lower in rural areas compared with urban areas (Ward *et al.* 2006). Spatial analyses have also identified reduced capacity of dead crow density measures to forecast human infections in rural areas (Eidson *et al.* 2005). These effects are seen because the capacity of crows to act as useful sentinels depends upon the likelihood that bird deaths are observed and reported by people. The power of dead crow sentinel surveillance to predict human risk is greatly reduced in rural areas as a consequence of a reduced detectability of the sentinel response.

purposefully designed to evaluate their potential. The major exception is the extensive research that has been carried out into the use of animal sentinels in the surveillance of West Nile virus (WNV) in North America (see box 1). The utility of different animal sentinel populations varies enormously according to both the ecological context and the aim of the surveillance programme, and in many cases animal sentinels may not prove a useful surveillance tool.

2. IDENTIFYING AND ASSESSING ANIMAL SENTINELS

For any population to be useful for surveillance, it must be under observation and must be capable of developing a detectable response to a particular pathogen. Sentinel populations are distinguished from other populations by having attributes that enhance detection of the disease or of the etiological agent and/or improve the

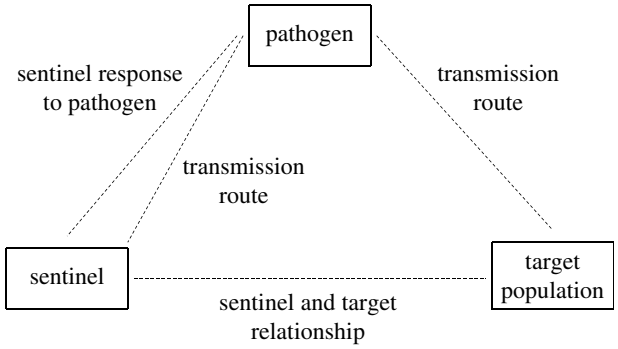


Figure 1. Key components and attributes of the sentinel framework.

cost-effectiveness of surveillance (McCluskey 2003). In most cases, this means that the sentinel population is more likely to be exposed to, or to respond to the pathogen than other populations. This sentinel concept encompasses the variety of uses described above and can refer to any level of grouping from an individual to a larger unit, such as a herd or even a species.

Various authors have compiled lists of attributes of an 'ideal' sentinel (CAMEH 1991; Komar 2001), but these have invariably been created with a particular sentinel application in mind and there exists little or no consensus about the common characteristics or defining features of 'the sentinel'. This ambiguity, of course, reflects the fact that there is no innate quality of sentinel suitability that particular species or populations have. Instead, the criteria against which the usefulness of a given sentinel population is assessed are influenced by the aim of surveillance and the context in which the sentinel would be used. We describe a conceptual framework which we believe can be used to evaluate potential sentinel populations for any combination of surveillance aim and ecological context (figures 1 and 2).

3. THE SENTINEL FRAMEWORK

Within any surveillance context, the sentinel population must always interact with both the pathogen and the target population and it is essential to consider and describe the interactions between these fundamental components (figure 1). The following are the three components of the sentinel framework.

— *Pathogen*. The pathogen that is under surveillance.
— *Target population*. The population of concern to which information gathered from the sentinel is applied.
— *Sentinel population*.

This framework is not intended to represent the transmission dynamics of a pathogen, but rather the ways in which the components are associated. Three critical attributes of this system must be considered in order to assess the utility of a potential sentinel for a particular surveillance aim and in any given ecological context: (i) the sentinel response to the pathogen, (ii) the relationship between sentinel and target populations, and (iii) routes of transmission to both target and sentinel populations. The conceptual issues raised are discussed with reference to the surveillance of WNV in North America (box 1).

3.1. Sentinel response to pathogen

The sentinel response to a pathogen can range from the production of antibody in an otherwise healthy individual, through morbidity and ultimately to mortality. It may also be possible to detect the presence of the pathogen in a sentinel population before other responses develop and sentinel responses can therefore include the following.

— Current infection/presence of pathogen.
— Seroconversion.
— Morbidity.
— Mortality.

There is a clear intuitive distinction between sentinel populations that develop high levels of morbidity or mortality in response to pathogen exposure and those that remain healthy. Sick or dying sentinels show an obvious and dramatic response to a pathogen and provide a readily appreciable signal of the presence of a pathogen within an ecosystem (see the discussion of crow mortality as a sentinel of WNV presence in box 1). At the other end of the spectrum, apparently healthy sentinels that develop a subclinical response are often more useful for investigating the maintenance patterns and transmission dynamics of a pathogen within the sentinel and target populations. Following the consumption of prey infected with rabbit haemorrhagic disease virus (RHDV), foxes in northern Germany developed antibody responses that declined after just two weeks. Serosurveillance of this fox population therefore reveals the proportion of the population that has been exposed in the one to two weeks prior to testing. These serological data can provide a good indication of the incidence patterns of RHDV in the sympatric rabbit population (Frölich *et al.* 1998). In cases in which healthy sentinels are used, it may be desirable to resample the same individuals or populations over time. It is also important that the observation and sampling of the sentinel population, and perhaps also the sentinel response itself, has minimal impact upon the study system.

This example also demonstrates the influence of the temporal characteristics of the sentinel response to a pathogen upon the choice and application of sentinel populations. Sentinel populations which respond to a pathogen prior to the exposure of the target population may be useful for those surveillance programmes that aim to prevent the exposure of the target population. For other sentinel uses, the rapid development of a response may not be required. The duration of the potential sentinel's response can also influence the types of question which can be usefully addressed. An equivalent sentinel population (to that of the foxes) that developed a longer lasting antibody response in the above RHDV example would be of limited use for investigating the incidence of disease in the rabbit population on this immediate time-scale.

The sentinel response can be viewed as a test for the presence of the pathogen within the target population

and as such has properties that are analogous to test sensitivity and specificity.

— *Sentinel sensitivity.* The sensitivity of the sentinel refers to its capacity to respond to the presence of the pathogen in the target population and effectively translates as susceptibility to infection. An insensitive population would be unlikely to display evidence of infection with the pathogen even if it were present in the target population and would therefore be poorly suited for use as a sentinel.
— *Sentinel specificity.* The specificity of the sentinel response relates to the ease with which a sentinel response can be interpreted and attributed to a particular pathogen. Specificity is thus closely linked to the response type. Morbidity and mortality are generally less specific indicators of the presence of a particular pathogen than molecular responses that are observed using a test or assay unique to the pathogen in question. Across parts of rural Africa and Asia, for example, bird die-offs due to pathogens other than H5N1 avian influenza virus can be relatively common occurrences, reducing the specificity of bird mortality as an indicator of H5N1 presence (World Health Organization 2005).

Whatever the type of response a particular sentinel population mounts to a pathogen, it is important that the individual members of that population are consistent in the development of the response. Excessive variation within a sentinel population would greatly complicate the interpretation of surveillance findings and it may therefore be important to ensure that members of the sentinel population are of similar age, sex or other relevant characteristics, depending upon the type of response measured.

3.2. Relationship between sentinel and target populations

The relationship that exists between the sentinel and target populations may include behavioural, epidemiological or spatial aspects or any other form of ecological association. Detailed understanding of the associations between the sentinel and target populations is not required to address all questions. However, a comprehensive understanding of the relationship between a sentinel and a target population will allow for the investigation of more complex epidemiological questions and better-informed interpretation of the data collected through surveillance of that sentinel. The minimum association that must exist between a sentinel and a target population is a spatial association. This need not imply spatial overlap however. If the pathogen is spreading on a wavefront or emanating from a focal source, then a sentinel population may be selected on the basis of its closer proximity to the focus at the target population.

At the other extreme, the sentinel population may consist of a specific subset of the target population, ensuring a very close relationship between the two populations. A subpopulation that experiences high-transmission risk, or is particularly sensitive to infection with a particular pathogen, may serve as a sentinel for the wider population and can clearly provide a more accurate assessment of risk to the target than a population occupying a dissimilar ecological niche and consequently experiencing a very different pattern of exposure to the pathogen (e.g. unvaccinated sentinel birds are used to detect the presence of HPAI viruses within the otherwise vaccinated flock; Suarez 2005). The sentinel and target population may also be epidemiologically linked such that the sentinel may act as a source of infection for the target population, as is the case with arthropod vector surveillance.

3.3. Transmission routes

This attribute is essentially a component of the relationship between the sentinel and target populations that explicitly considers the route or routes through which the two populations can become infected with the pathogen. In circumstances where the target and sentinel are exposed to infection via the same route, the relative intensity and patterns of exposure of the two populations to the source of infection are important (Estrada-Franco *et al.* 2006). It may be desirable to select a sentinel that has higher levels of exposure and which is therefore more likely to show evidence of a pathogen if it is present than to directly survey the target population itself. For pathogens that are transmitted by a vector or vectors, the feeding preferences of the vector(s) can therefore be important in informing sentinel selection. Domestic dogs are the preferred source of blood meals for *Triatoma infestans*, one of the main vectors of *Trypanosoma cruzi* in Mexico. A comparative serosurvey revealed overall anti-*T. cruzi* IgG prevalence of 16% in dogs compared with a 2% prevalence in humans, and a strong positive correlation between human and dog seropositivity within the study area. These data suggest that the feeding preferences of this vector make the domestic dog population a good sentinel for identifying areas of human seropositivity and monitoring prevalence in this context (Estrada-Franco *et al.* 2006).

There are also circumstances in which the route of exposure of the sentinel and target population may differ. A number of emerging zoonoses, including WNV and HPAI H5N1 viruses can be transmitted through the ingestion of infected material (Komar *et al.* 2003; Austgen *et al.* 2004; Rimmelzwaan *et al.* 2006). Carnivore and scavenger species that are exposed through consumption of infected prey may prove useful sentinels for a wide range of pathogens, specifically because of this additional route of exposure that is not shared with the target population (Cleaveland *et al.* 2006). A single predator or scavenger typically consumes material from multiple individuals, increasing the probability of exposure to pathogens circulating within the prey population. Predators and scavengers can effectively sample from the prey population, leading to a 'bioaccumulation' effect whereby pathogens present at relatively low prevalence in the prey population may be detected at higher prevalence in the predator/scavenger species (Cleaveland *et al.* 2006). An understanding of the predator–prey relationships between the target population and potential sentinels may prove useful in sentinel selection. The principal

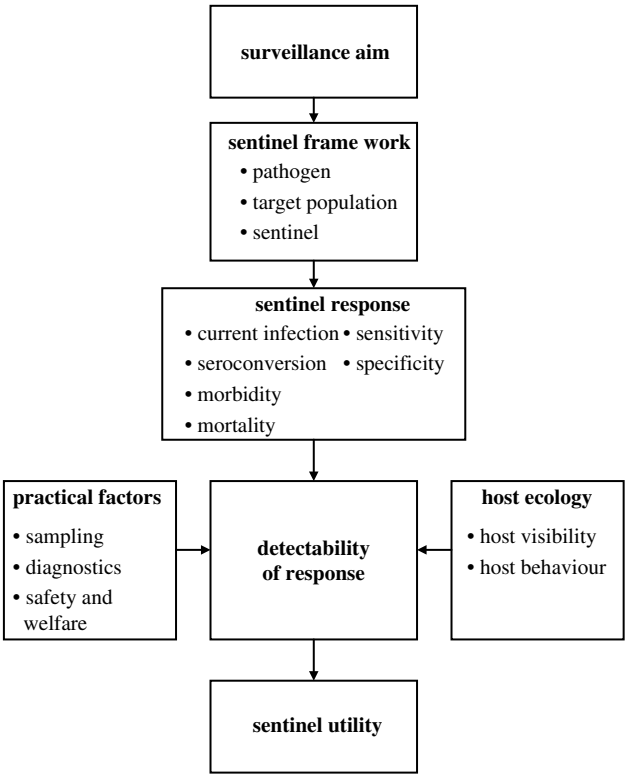


Figure 2. The sentinel framework in context.

transmission route of bluetongue virus, which infects wild and domestic ruminants across East Africa, is via *Culicoides* midge vectors. Serosurveillance of free-ranging African carnivores revealed that both the seroprevalence and the virus serotype identified varied dramatically across carnivore species (Alexander *et al.* 1994). This study suggested that the most probable route of infection of carnivores with bluetongue was via consumption of infected prey, and that the variation seen between species was attributable to dietary differences. Different carnivore species may therefore vary in their utility as sentinels for the presence of bluetongue virus in different ruminant species.

4. PLACING THE SENTINEL FRAMEWORK IN CONTEXT

The sentinel response can be viewed as the output of the sentinel framework. The nature of this response, in combination with other sentinel host factors and practical influences which depend upon the context in which surveillance is conducted, determines the overall detectability of the sentinel response (figure 2). Unlike the attributes which operate within the sentinel framework, detectability is a quality of the interaction between the sentinel and the observer. The overall utility of any potential sentinel can only be assessed by considering both the sentinel framework and the influences of the context in which it would be applied (figure 2; box 2).

The visibility of any animal population is determined by the morphology, behaviour, distribution and abundance of the individual animals of which it is comprised. The detectability of the sentinel response includes both the visibility of the animal and its response to a pathogen. The type of response that an animal mounts will directly affect the ease with which it is detected by the observer. For example, lions are being used as sentinels for canine distemper in the Serengeti National Park in Tanzania, as a result of their high visibility to observers and the dramatic manifestations of clinical disease, which include grand mal seizures (Roelke-Parker *et al.* 1996). Information from lion sentinels would be used to increase disease detection efforts within other wild carnivore populations of the park to establish the extent and impact of any epidemic and initiate a risk–benefit assessment for possible interventions (such as vaccination) for protecting threatened wildlife populations. A wide range of other carnivore species such as hyaenas, bat-eared foxes and leopards are known to be susceptible to canine distemper (Roelke-Parker *et al.* 1996), but are less suitable as sentinels for disease in the Serengeti owing to ecological and behavioural factors that reduce visibility (e.g. nocturnal behaviour, small body size, den-living characteristics, lower levels of tourist observation). Widespread morbidity or mortality within a sentinel population are more readily appreciable than seroconversion or current infection/presence of pathogen, which can only be detected by the observer after first sampling the sentinel population and then conducting laboratory analysis. In the case of overt sentinel responses such as mortality, the existence of a reliable network of 'observers' and a mechanism through which data are reported are crucial. It is equally important to consider the available capacity to detect any less overt responses including the existence of a reliable sampling protocol and a diagnostic test (McCluskey 2003). The majority of diagnostic tests for human and livestock pathogens have not been validated for use in non-target species and the sensitivity and specificity of tests can vary hugely between species (Greiner & Gardner 2000). The existence of a suitable negative control population and recognition of the time required to identify and validate diagnostic tests must be considered in any proposed sentinel surveillance programme.

The practical difficulties involved in sampling any potential sentinel population must also be evaluated and it may often be difficult to reconcile the use of a theoretically ideal sentinel with such practicalities. For a sentinel population to be useful, it must be both logistically feasible and safe to sample sufficient numbers of the population (CAMEH 1991). Since sentinels are often selected on the basis of increased likelihood of exposure to a pathogen, sentinel surveillance can enable targeting of resources and often has improved cost-effectiveness as compared, for example, with more comprehensive cross-sectional surveys (McCluskey *et al.* 2003). The bioaccumulation effect discussed above suggests that evidence of exposure to a pathogen may effectively accumulate within carnivore populations (Cleaveland *et al.* 2006). The identification of the presence of a pathogen within a particular area can therefore be achieved by sampling relatively few carnivore sentinels, as compared to an exhaustive and costly survey of the prey population

Box 2. Simplified application of the conceptual framework represented in figures 1 and 2 to the evaluation of potential sentinel populations for the surveillance of HPAI H5N1.

Surveillance aim

To establish if H5N1 viruses have been introduced into a country with underdeveloped disease surveillance and reporting structure.

Should sentinels be used?
— Cross-sectional survey–may be expensive and time consuming.
— Sentinel surveillance–potentially cost-effective alternative

↓

Sentinel framework

pathogen = HPAI H5N1 virus.
target population = the national poultry population.
Potential sentinels
— Backyard chicken populations in areas of perceived high risk of virus introduction, e.g. close to areas of wild bird congregation or to livestock markets.
— Backyard ducks in similar locations.
— Wild bird populations.
— Domestic cats.
— Domestic dogs.

Other potential sentinels are excluded altogether on the basis of a lack of response to the pathogen or of any type of meaningful relationship with the target population.

Relationship between sentinel and target populations

Chickens
— Subset of target population.
Ducks
— Occupy a very similar niche to target population.
— May act as silent carrier of viruses (Hulse-Post *et al.* 2005).
Wild birds
— May act as source of infection for domestic species.
— May not occupy the same geographical areas as the target population (especially true for large congregations of migratory birds).
Cats and dogs
— Spatial correspondence with target population.
— Cats and dogs may prey upon the target population.

Transmission routes

Chickens, ducks and wild birds
— Bird–bird transmission.
— Environmental contamination.
Cats and dogs
— Consumption of infected birds (Keawcharoen *et al.* 2004; Kuiken *et al.* 2004).
— Horizontal transmission in cats (Rimmelzwaan *et al.* 2006).

↓

Sentinel response

Chickens
— Consistent, rapid and widespread mortality.
— Die-offs provide a prompt indication of virus presence.
Ducks
— Variable pathogenicity and thus mortality (Sturm-Ramirez *et al.* 2005).
— Isolation of virus from healthy birds (Hulse-Post *et al.* 2005).
Wild birds
— Variable pathogenicity (Ellis *et al.* 2004).
— Isolation of virus from healthy birds (Chen *et al.* 2006).
Cats
— Experimental evidence of mortality response (Rimmelzwaan *et al.* 2006).
— Mortality reports associated with bird die-offs (Butler 2006*a*, Songserm *et al.* 2006*a*, Yingst *et al.* 2006).
— High seroconversion rates (Butler 2006*b*).
— Subclinical infections (Leschnik *et al.* 2007).
Dogs
— High seroconversion rates (Butler 2006*b*).
— Mortality report associated with bird infection (Songserm *et al.* 2006*b*).

Sensitivity and specificity of responses

Chickens
— ✓✓✓ Highly sensitive but specificity of mortality response is low, as
— ××× chicken die-offs not necessarily unusual where poultry are not routinely vaccinated against other pathogens,

(*Continued.*)

Box 2. (*Continued.*)

e.g. Newcastle disease virus.
— ✓✓✓ High specificity of laboratory analyses.

Ducks
— ✓/× Variable mortality response limits sensitivity.
— ✓✓✓ High specificity of laboratory analyses.

Wild birds
— ✓/× Variable mortality response limits sensitivity.
— ××× Very low prevalence in healthy birds limits sensitivity (Chen *et al.* 2006).
— ✓✓✓ High specificity of laboratory analyses.

Cats and Dogs
— ✓/× Serological analyses non-specific for distinguishing high- and low-pathogenicity viruses.

+

Host ecology

— ✓✓✓ Domestic species are all highly observable as a consequence of their close association with humans.
— ××× Wild birds are considerably less visible and may occupy relatively remote and inaccessible areas.

+

Practical factors

— Risk to sampling personnel must be considered as a priority when developing all sampling protocols.
— ✓✓✓ Domestic species approachable and handleable.
— ✓/× Distribution of cats and dogs relatively to poultry may vary according to factors such as urbanization and religion.
— ××× Considerable investment of money, time and expertise required to sample sufficient numbers of wild birds.
— ✓✓✓ For the identification of virus presence, standard test protocols include RT-PCR and virus isolation (World Organization for Animal Health 2005) which are generally adaptable across species.
— ✓/× Serological analyses may not be well developed for wild birds, cats or dogs.

↓

Detectability

Chickens
— ✓✓✓ Mortality response easily appreciated.
— ✓✓✓ High visibility within human communities.
— ××× Low specificity of mortality limits detectability.

Ducks
— ××× Mortality response variable.
— ✓✓✓ Additional responses detectable through laboratory analysis.
— ✓✓✓ High visibility within human communities.

Wild birds
— ××× Mortality response variable.
— ××× Low visibility compared with domestic species.
— Logistically complex and time-consuming sampling required.

Cats and Dogs
— ✓✓✓ High visibility within human communities.
— ✓✓✓ Sudden and widespread morbidity or mortality uncommon.
— ✓/× Non-mortality responses less detectable.

In all cases, a comprehensive network of observers is vital and it may be necessary to develop education programmes aimed at improving reporting levels.

↓

Utility

— Domestic chicken and ducks sentinels are likely to provide the most rapid and dramatic response to HPAI H5N1 virus within a country. However, in this context in which mortality in domestic birds is not unusual, this mortality may not be reported and the detectability of the response in the context of this surveillance aim may be very low.
— To best address this surveillance aim, the specificity of the chicken mortality response to HPAI H5N1 presence could be enhanced by using a combination of sentinels such that priority was given to the investigation of chicken die-offs that were accompanied by morbidity or mortality in cats or dogs (Yingst *et al.* 2006).
— Retrospective analysis of sera collected from ducks, cats and dogs could also be used to identify those areas in which an H5N1 virus had been present.

within which the pathogen may circulate at very low prevalence, thereby providing a relatively rapid and inexpensive surveillance option (Frölich *et al.* 1998; Leighton *et al.* 2001; Csángó *et al.* 2004; Cleaveland *et al.* 2006). In addition to consideration of time and cost, the potential risks to research personnel and the public that are associated with the desired sampling strategy must be evaluated, as well as the effects of sampling upon the sentinel population itself in the context of animal welfare and conservation status (CAMEH 1991).

5. APPLICATIONS OF ANIMAL SENTINELS

Many of the questions addressed through the use of animal sentinels, such as the assessment of pathogen

control efforts, the monitoring of prevalence fluctuations over time and the demonstration of the absence of a pathogen, require only the basic qualities of a sentinel as defined above. While the more specific requirements of any particular sentinel are unique to the context and aim to which it is applied, there are some general qualities and subtypes of sentinels that correspond to major applications of animal sentinels. For example, sentinels in which the response to a pathogen and the detection of that response occur prior to exposure, or cases in the target population, can provide early warning of pathogen presence. Early warning sentinels are used to provide a predictive signal of risk to the target population. Sentinels that are exposed and which respond to a pathogen before the exposure of the target population may provide an opportunity to implement pre-emptive control measures and to prevent the infection of the target population (see discussion of WNV surveillance in box 1). Other early warning sentinels may respond to the pathogen more rapidly than the target population but not necessarily before the target's exposure (e.g. the coal miner's canary). In such cases, data collected from the sentinel cannot be used to prevent cases in the target population altogether. However, the information they supply can provide advance warning of cases, enabling the prioritization of resources for treatment and the prevention of additional cases. In most cases, early warning sentinels are highly visible and develop a very obvious response to the pathogen. Data provided by sentinels with these qualities can be more rapidly processed, analysed and acted upon than the data from apparently healthy sentinels for which the potentially lengthy processes of sample collection and laboratory analyses must be carried out before any data are available. Ideally, the response of early warning sentinels should also be very specific to minimize the likelihood of false positive responses and consequently improve confidence in decision making based on the sentinel response alone.

Sentinels can also be used retrospectively to provide evidence of the timing of pathogen introduction and spread through a target population. In situations where a number of populations or locations are sampled, this information can be combined to reveal the spatial and temporal pattern of pathogen spread. Following the widespread rinderpest outbreak that occurred in Kenya in 1993–1997, the retrospective serosurveillance of buffalo herds and analysis of age-seroprevalence patterns allowed the estimation of the time of infection in different herds, the identification of the probable point of entry of the pathogen into the wildlife population and the elucidation of where the pathogen had been, how it had spread and where it was likely to move to (Kock *et al.* 1999). In this case, buffalo herds were selected as sentinels on the basis of the increased susceptibility of the species to this virus (Rossiter 1994), and served as sentinels for the larger livestock population in the affected areas. In such circumstances, the appropriate sentinel population must develop a response to the pathogen that persists and is detectable a long time after exposure. When used retrospectively, it is also important that individuals of the sentinel population can be reliably aged.

6. CONCLUSION

The objective of this paper has been to provide a consistent and inclusive framework that clarifies our understanding of the role of animal sentinels and their potential value in the surveillance of human and animal infectious diseases, as well as providing a conceptual tool that can be applied to assess and characterize potential sentinels in the future. At present, surveillance of many pathogens involves the target population alone; however, the broad host range of many important human and animal diseases provides opportunities for exploiting a wide range of species for surveillance purposes. The variability of host responses to a pathogen, the heterogeneities in pathogen exposure in different populations and the differing relationships between sentinel and target populations indicate that different animal hosts will themselves vary in their ability to act as effective sentinels in different circumstances.

Animal sentinels may not serve as a useful surveillance tool in all contexts. The generic framework that we have developed in this paper describes the attributes of host species that need to be considered to identify appropriate sentinel populations for different surveillance purposes. This same framework should also be used to identify characteristics of potential sentinels that perhaps make them unsuitable in a particular circumstance. For example, sentinels must by definition be intentionally observed. This classification distinguishes the use of animal sentinels from scenarios in which responses of animal populations to novel pathogens are 'noticed'. For this reason, animal sentinels cannot provide the solution to the question of how to carry out surveillance for pathogens that are currently unknown. However, as a consequence of greater awareness of the potential of animal sentinels and improved observation of animal populations, instances of unusual morbidity and mortality in animal populations that result from the emergence of novel pathogens would perhaps be more likely to be noticed and their potential significance to other species recognized.

To date, there has been limited appreciation of the data resource that different animal hosts represent for disease surveillance. This paper aims to highlight the variety of surveillance functions for which animal sentinels may be used, the range of animal host species that may usefully be exploited (particularly for human disease surveillance) and the potential benefits of animal sentinels for enhanced pathogen detection and improved cost-effectiveness of surveillance. The potential value of animal sentinels in disease prevention and control can only be realized with close integration and effective communication between and within human and animal health sectors; information generated from sentinel populations must be disseminated to those who need to

take action, and appropriate responses must be generated as a result of this information to mitigate disease risk.

We acknowledge the following agencies for supporting components of this work: the Wellcome Trust, Department for the Environment, Food and Rural Affairs and NIH/NSF Ecology of Infectious Diseases Program (grant no. 0225453). Any opinions, findings, conclusions or recommendations expressed are those of the authors and do not necessarily reflect the views of the National Science Foundation. We would also like to thank two anonymous reviewers for very helpful comments on earlier versions of the manuscript and the members of the Wildlife and Emerging Diseases Section, R(D)SVS, University of Edinburgh, for their many contributions that led to the development of the concepts outlined in this article.

REFERENCES

Alexander, K. A. *et al.* 1994 Evidence of natural bluetongue virus-infection among African carnivores. *Am. J. Trop. Med. Hyg.* **51**, 568–576.

Austgen, L. E., Bowen, R. A., Bunning, M. L., Davis, B. S., Mitchell, C. J. & Chang, G.-J. J. 2004 Experimental infection of cats and dogs with West Nile virus. *Emerg. Infect. Dis.* **10**, 82–86.

Burrell, G. A. & Seibert, F. M. 1916 *Gases found in coal mines. Miner's circular*, vol. 14. Washington, DC: Bureau of Mines, Department of the Interior.

Butler, D. 2006*a* Can cats spread avian flu? *Nature* **440**, 135–135. (doi:10.1038/440135a)

Butler, D. 2006*b* Thai dogs carry bird-flu virus, but will they spread it? *Nature* **439**, 773–773. (doi:10.1038/439773a)

Campbell, G. L., Marfin, A. A., Lanciotti, R. S. & Gubler, D. J. 2002 West Nile virus. *Lancet Infect. Dis.* **2**, 519–529. (doi:10.1016/S1473-3099(02)00368-7)

Centers for Disease Control and Prevention 2002 Manual for the surveillance of vaccine-preventable diseases, 3rd edn. See http://www.cdc.gov/nip/publications/surv-manual/default.htm.

Chen, H. *et al.* 2006 Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. *Proc. Natl Acad. Sci. USA* **103**, 2845–2850. (doi:10.1073/pnas.0511120103)

Chomel, B. B. 2003 Control and prevention of emerging zoonoses. *J. Vet. Med. Educ.* **30**, 145–147.

Cleaveland, S., Meslin, F. X. & Breiman, R. 2006 Dogs can play useful role as sentinel hosts for disease. *Nature* **440**, 605. (doi:10.1038/440605b)

Committee on Animals as Monitors of Environmental Hazards 1991 *Animals as sentinels of environmental health hazards.* Washington, DC: National Academy Press.

Csángó, P. A., Blakstad, E., Kirtz, G. C., Pedersen, J. E. & Czettel, B. 2004 Tock-borne encephalitis in southern Norway. *Emerg. Infect. Dis.* **10**, 533–534.

Dugan, V. G., Yabsley, M. J., Tate, C. M., Mead, D. G., Munderloh, U. G., Herron, M. J., Stallknecht, D. E., Little, S. E. & Davidson, W. R. 2006 Evaluation of white-tailed deer (*Odocoileus virginianus*) as natural sentinels for *Anaplasma phagocytophilum*. *Vector-Borne Zoonot. Dis.* **6**, 192–207. (doi:10.1089/vbz.2006.6.192)

Eidson, M., Komar, N., Sorhage, F., Nelson, R., Talbot, T., Mostashari, F. & Mclean, R. 2001*a* Crow deaths as a sentinel surveillance system for West Nile virus in the Northeastern United States, 1999. *Emerg. Infect. Dis.* **7**, 615–620.

Eidson, M., Kramer, L., Stone, W., Hagiwara, Y. & Schmit, K. 2001*b* Dead bird surveillance as an early warning system for West Nile virus. *Emerg. Infect. Dis.* **7**, 631–635.

Eidson, M., Schmit, K., Hagiwara, Y., Anand, M., Backenson, P. B., Gotham, I. & Kramer, L. 2005 Dead crow density and West Nile virus monitoring, New York. *Emerg. Infect. Dis.* **11**, 1370–1375.

Ellis, T. M. *et al.* 2004 Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. *Avian Pathol.* **33**, 492–505. (doi:10.1080/03079450400003601)

Estrada-Franco, J. G., Bhatia, V., Diaz-Albiter, H., Ochoa-Garcia, L., Barbabosa, A., Vazquez-Chagoyan, J. C., Martin-Perez, M. A., Guzman-Bracho, C. & Garg, N. 2006 Human *Trypanosoma cruzi* infection and seropositivity in dogs, Mexico. *Emerg. Infect. Dis.* **12**, 624–630.

Ferguson, N. M., Cummings, D. A. T., Cauchemez, S., Fraser, C., Riley, S., Meeyai, A., Iamsirithaworn, S. & Burke, D. S. 2005 Strategies for containing an emerging influenza pandemic in Southeast Asia. *Nature* **437**, 209–214. (doi:10.1038/nature04017)

Frölich, K., Klima, F. & Dedek, J. 1998 Antibodies against rabbit hemorrhagic disease virus in free-ranging red foxes from Germany. *J. Wildl. Dis.* **34**, 436–442.

Glickman, L. T., Domanski, L. M., Maguire, T. G., Dubielzig, R. R. & Churg, A. 1983 Mesothelioma in pet dogs associated with exposure of their owners to asbestos. *Environ. Res.* **32**, 305–313. (doi:10.1016/0013-9351(83)90114-7)

Greiner, M. & Gardner, I. A. 2000 Epidemiologic issues in the validation of veterinary diagnostic tests. *Prev. Vet. Med.* **45**, 3–22. (doi:10.1016/S0167-5877(00)00114-8)

Hayes, E. B. & Gubler, D. J. 2006 West Nile virus: epidemiology and clinical features of an emerging epidemic in the United States. *Annu. Rev. Med.* **57**, 181–194. (doi:10.1146/annurev.med.57.121304.131418)

Hulse-Post, D. J. *et al.* 2005 Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. *Proc. Natl Acad. Sci. USA* **102**, 10 682–10 687. (doi:10.1073/pnas.0504662102)

Institute of Laboratory Animal Resources (US). Committee on infectious diseases of mice and rats 1991 *Infectious diseases of mice and rats.* Washington, DC: National Academy Press.

Johnson, G. D., Eidson, M., Schmit, K., Ellis, A. & Kulldorff, M. 2006 Geographic prediction of human onset of West Nile virus using dead crow clusters: an evaluation of year 2002 data in New York State. *Am. J. Epidemiol.* **163**, 171–180. (doi:10.1093/aje/kwj023)

Kahn, L. H. 2006 Confronting zoonoses, linking human and veterinary medicine. *Emerg. Infect. Dis.* **12**, 556–561.

Keawcharoen, J. *et al.* 2004 Avian influenza H5N1 in tigers and leopards. *Emerg. Infect. Dis.* **10**, 2189–2191.

Kile, J. C., Panella, N. A., Komar, N., Chow, C. C., Macneil, A., Robbins, B. & Bunning, M. L. 2005 Serologic survey of cats and dogs during an epidemic of West Nile virus infection in humans. *J. Am. Vet. Med. Assoc.* **226**, 1349–1353. (doi:10.2460/javma.2005.226.1349)

Kilpatrick, A. M., Kramer, L. D., Campbell, S. R., Alleyne, E. O., Dobson, A. P. & Daszak, P. 2005 West Nile virus risk assessment and the bridge vector paradigm. *Emerg. Infect. Dis.* **14**, 425–429.

Kilpatrick, A. M., Kramer, L. D., Jones, M. J., Marra, P. P. & Daszak, P. 2006 West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. *PLoS Biol.* **4**, 606–610. (doi:10.1371/journal.pbio.0040082)

Kock, R. A., Wambua, J. M., Mwanzia, J., Wamwayi, H., Ndungu, E. K., Barrett, T., Kock, N. D. & Rossiter, P. B. 1999 Rinderpest epidemic in wild ruminants in Kenya 1993–97. *Vet. Rec.* **145**, 275–283.

Komar, N. 2001 West Nile virus surveillance using sentinel birds. In *West Nile virus: detection, surveillance, and control* (eds D. J. White & D. L. Morse), pp. 58–73. New York, NY: Academy of Sciences.

Komar, N., Panella, N. A. & Boyce, E. 2001 Exposure of domestic mammals to West Nile virus during an outbreak of human encephalitis, New York City, 1999. *Emerg. Infect. Dis.* **7**, 736–738.

Komar, N., Langevin, S., Hinten, S., Nemeth, N., Edwards, E., Hettler, D., Davis, B., Bowen, R. & Bunning, M. 2003 Experimental infection of north American birds with the New York 1999 strain of West Nile virus. *Emerg. Infect. Dis.* **9**, 311–322.

Kuiken, T., Rimmelzwaan, G., van Riel, D., van Amerongen, G., Baars, M., Fouchier, R. & Osterhaus, A. 2004 Avian H5N1 influenza in cats. *Science* **306**, 241. (doi:10.1126/science.1102287)

Kuiken, T., Leighton, F. A., Fouchier, R. A. M., Leduc, J. W., Peiris, J. S. M., Schudel, A., Stohr, K. & Osterhaus, A. 2005 Public health—pathogen surveillance in animals. *Science* **309**, 1680–1681. (doi:10.1126/science.1113310)

Leighton, F. A., Artsob, H. A., Chu, M. C. & Olson, J. G. 2001 A serological survey of rural dogs and cats on the southwestern Canadian prairie for zoonotic pathogens. *Can. J. Public Health* **92**, 67–71.

Leschnik, M. *et al.* 2007 Subclinical infection with avian influenza A (H5N1) virus in cats. *Emerg. Infect. Dis.* **13**, 243–247.

Loftin, K. C. *et al.* 2006 Five-year surveillance of West Nile and Eastern equine encephalitis viruses in southeastern Virginia. *J. Environ. Health* **68**, 33–40.

McCluskey, B. J. 2003 Use of sentinel herds in monitoring and surveillance systems. In *Animal disease surveillance and survey systems: methods applications* (ed. M. D. Salman), pp. 119–133. Iowa, IA: Iowa State Press.

McCluskey, B. J., Mumford, E. L., Salman, M. D. & Traub-Dargatz, J. J. 2002 Use of sentinel herds to study the epidemiology of vesicular stomatitis in the state of Colorado. In *Domestic animal/wildlife interface: issue for disease control, conservation, sustainable food production, and emerging diseases* (eds E. P. J. Gibbs & R. H. Bokma), pp. 205–209. New York, NY: Academy of Sciences.

McCluskey, B. J., Burgess, B., Glover, J., Kinde, H. & Hietala, S. 2006 Use of sentinel chickens to evaluate the effectiveness of cleaning and disinfection procedures in noncommercial poultry operations infected with exotic Newcastle disease virus. *J. Vet. Diag. Invest.* **18**, 296–299.

McLean, R. G., Ubico, S. R., Docherty, D. E., Hansen, W. R., Sileo, L. & Mcnamara, T. S. 2001 West Nile virus transmission and ecology in birds. In *West Nile virus: detection, surveillance, and control* (eds D. J. White & D. L. Morse), pp. 54–57. New York, NY: Academy of Sciences.

Molaei, G., Andreadis, T. A., Armstrong, P. M., Anderson, J. F. & Vossbrinck, C. R. 2006 Host feeding patterns of Culex mosquitoes and West Nile virus transmission, northeastern United States. *Emerg. Infect. Dis.* **12**, 468–474.

Mostashari, F. *et al.* 2001 Epidemic West Nile encephalitis, New York, 1999: results of a household-based seroepidemiological survey. *Lancet* **358**, 261–264. (doi:10.1016/S0140-6736(01)05480-0)

Mostashari, F., Kulldorff, M., Hartman, J. J., Miller, J. R. & Kulasekera, V. 2003 Dead bird clusters as an early warning system for West Nile virus activity. *Emerg. Infect. Dis.* **9**, 641–646.

National Arbovirus Monitoring Program 2003–2004 NAMP annual report. See http://www.namp.com.au/reports/report_0304.pdf.

Nugent, G., Whitford, J. & Young, N. 2002 Use of released pigs as sentinels for *Mycobacterium bovis*. *J. Wildl. Dis.* **38**, 665–677.

Rabinowitz, P. M., Gordon, Z., Holmes, R., Taylor, B., Wilcox, M., Chudnov, D., Nadkarni, P. & Dein, F. J. 2005 Animals as sentinels of human environmental health hazards: an evidence-based analysis. *EcoHealth* **2**, 26–37. (doi:10.1007/s10393-004-0151-1)

Rimmelzwaan, G. F., van Riel, D., Baars, M., Bestebroer, T. M., van Amerongen, G., Fouchier, R. A. M., Osterhaus, A. D. M. E. & Kuiken, T. 2006 Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. *Am. J. Pathol.* **168**, 176–183. (doi:10.2353/ajpath.2006.050466)

Roelke-Parker, M. E. *et al.* 1996 A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). *Nature* **379**, 441–445. (doi:10.1038/379441a0)

Rossiter, P. B. 1994 Rinderpest. In *Infectious diseases of lifestock* (eds J. A. W. Coetzer, G. R. Thomson & R. C. Tustin), pp. 735–757. Oxford, UK: Oxford University Press.

Rouquet, P. *et al.* 2005 Wild animal mortality monitoring and human Ebola outbreaks, Gabon and Republic of Congo, 2001–2003. *Emerg. Infect. Dis.* **11**, 283–290.

Salman, M. D. 2003 Surveillance and monitoring systems for animal health programs and disease surveys. In *Animal disease surveillance and survey systems: methods and applications* (ed. M. D. Salman), pp. 3–13. Iowa, IA: Iowa State Press.

Schwabe, C. W. 1984 *Veterinary medicine and human health*. Baltimore, MD: Williams and Wilkins.

Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Meemak, N., Pariyothorn, N., Payungporn, S., Theamboonlers, A. & Poovorawan, Y. 2006*a* Avian influenza H5N1 in naturally infected domestic cat. *Emerg. Infect. Dis.* **12**, 681–683.

Songserm, T. *et al.* 2006*b* Fatal avian influenza A H5N1 in a dog. *Emerg. Infect. Dis.* **12**, 1744–1747.

Sturm-Ramirez, K. M. *et al.* 2005 Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? *J. Virol.* **79**, 11 269–11 279. (doi:10.1128/JVI.79.17.11269-11279.2005)

Suarez, D. L. 2005 Overview of avian influenza DIVA test strategies. *Biologicals* **33**, 221–226. (doi:10.1016/j.biologicals.2005.08.003)

Thrusfield, M. V. 2005 *Veterinary epidemiology*, 3rd edn. Oxford, UK: Blackwell Science.

Ward, M. R., Stallknecht, D. E., Willis, J., Conroy, M. J. & Davidson, W. R. 2006 Wild bird mortality and West Nile virus surveillance: biases associated with detection, reporting, and carcass persistence. *J. Wildl. Dis.* **42**, 92–106.

Woolhouse, M. E. J. & Gowtage-Sequeria, S. 2005 Host range and emerging and reemerging pathogens. *Emerg. Infect. Dis.* **11**, 1842–1847.

Work, T. H., Hurlbut, H. S. & Taylor, R. M. 1955 Indigenous wild birds of the Nile-Delta as potential West Nile virus circulating reservoirs. *Am. J. Trop. Med. Hyg.* **4**, 872–888.

World Health Organization 2001 Protocol for the evaluation of epidemiological surveillance systems WHO/EMC/

DIS/97.2. See http://www.who.int/vaccines-documents/DocsWord/word577.doc.

World Health Organization 2005 Avian influenza and the pandemic threat in Africa: risk assessment for Africa. See http://www.who.int/csr/disease/avian_influenza/riskassessmentAfrica/en/index.html.

World Organization for Animal Health 2005 Manual of diagnostic tests and vaccines for terrestrial animals. Ch. 2.7.12. Avian influenza. See http://www.oie.int/Eng/Normes/Mmanual/A_00037.htm.

Yaremych, S. A., Warner, R. E., Mankin, P. C., Brawn, J. D., Raim, A. & Novak, R. 2004 West Nile virus and high death rate in American crows. *Emerg. Infect. Dis.* **10**, 709–711.

Yingst, S. L., Saad, M. D. & Felt, S. A. 2006 Qinghai-like H5N1 from domestic cats, northern Iraq. *Emerg. Infect. Dis.* **12**, 1295–1297.

APPENDIX 3

ROYAL (DICK) SCHOOL OF VETERINARY STUDIES
UNIVERSITY OF EDINBURGH

EVALUATION OF CARNIVORES AND SCAVENGERS AS SENTINELS FOR NEW AND EMERGING DISEASE

Dear Cat Owner,

I am conducting a government-funded study at the Dick Vet investigating whether hunters such as cats and foxes can tell us about the presence of certain infectious diseases that may be present in the wild animals that they eat, such as rodents and birds. This is important because many of these infections, such as leptospirosis, can cause disease in man and domestic animals, and we are looking for more efficient ways of detecting their presence. We know that, for some diseases, if a cat eats a rodent or bird that is infected, that cat will develop an immune response (antibodies) to that disease, but will not become ill. As cats potentially can eat hundreds of rodents and birds in their lifetime, they effectively sample the rodent and bird population for us.

As part of this study, we need to examine blood samples from **cats that go outdoors and hunt**, to see if we can detect the antibodies produced by exposure to some of the diseases that might be present in their prey.

If your cat needs a blood sample for any reason, as indicated by your vet, I am asking that, if there is any blood left over after the necessary tests have been done, it might be donated for this study. No excess blood will be obtained purely for the purpose of the study; I will only be using any aliquot of blood that would otherwise be discarded as clinical waste.

If you agree, I need to get your written agreement and to ask you to answer a few simple questions about your cat on the attached form.

Thank you so much to you and your cat for participating and contributing to this important study.

Yours sincerely

Anna Meredith MA VetMB CertLAS DZooMed MRCVS
Head of Exotic Animal and Wildlife Service

ROYAL (DICK) SCHOOL OF VETERINARY STUDIES
CATS AS SENTINELS
CONSENT FORM

NAME OF OWNER:_____

ADDRESS (incl. postcode):_____

_____

NAME OF CAT:_____

AGE in years:_____

BREED:_____

SEX: Male Female Neutered? Yes No

HOW DO YOU KNOW YOUR CAT HUNTS?

Dead or live prey items brought into the house

Dead prey items seen outside

HOW OFTEN DOES YOUR CAT HUNT?

Occasionally (less than once a week)

Regularly (more than once a week)

WHAT DOES YOUR CAT <u>HUNT</u>? (tick any that apply):

Rodents Shrews Rabbits Birds

DOES YOUR CAT EAT WHAT IT HUNTS, AT LEAST SOME OF THE TIME? (e.g you see it eating prey, or partially eaten carcases) Yes No

IF YES, WHAT DOES YOUR CAT <u>EAT</u>? (tick any that apply):

Rodents Shrews Rabbits Birds

Declaration:
I give my informed consent that if there is any spare blood left over from the legitimate veterinary sample being taken by my vet (under the Veterinary Surgeon's Act), it can be used by Anna Meredith for scientific purposes.

Signed……..
Name (block capitals)…….

Date…….

APPENDIX 5.1

5.1.1 SDS-PAGE reducing buffer

0.5M Tris HCl pH 6.8	5ml
Glycerol	4ml
10% sodium dodecyl sulphate (SDS)	8ml
2-Mercaptoethanol	2ml
0.1% Bromophenol blue	1ml

5.1.2 SDS-PAGE gels:

12% separation gel:

Distilled water	3.35ml
1.5M Tris HCl pH 8.8	2.5ml
10% SDS	100µl
Acrylamide/Bis(30% stock)	4ml
10% ammonium persulfate	100µl
TEMED	10µl

4% stacking gel:

Distilled water	6.1ml
0.5M Tris HCl pH 6.8	2.5ml
10% SDS	100µl
Acylamide/Bis(30% stock)	1.3ml
10% ammonium persulfate	100µl
TEMED	10µl

5.1.3 Urea extraction buffer

10 millimoles Tris

0.2% CHAPS

8M urea

5.1.4 ELISA reagents

Reagent	Constituents
Dilution and blocking buffer	Distilled water with 20mM TRIS/HCl pH 7.5, 150mM NaCL, 0.5% Tween 80,4% BSA
Conjugate	10ml dilution buffer, 1 µl Protein A, 5 µl Protein G
Wash buffer	Distilled water with 250mM NaCl and 0.05% Tween 20
Carbonate buffer	0.1M NaHCO3 titrated to pH 9.6 with 0.1M Na2CO3

5.1.5 Reference antisera

Reference antisera were obtained from the Leptospira Reference Unit (Health Protection Agency (HPA), Hereford, UK) against strain numbers 1,2,3,4,7,8,9,11,12,14,15. Antisera for strains 5, 6, 10 and 13 were not available. Reference hyperimmune antisera were raised in rabbits after inoculation with a cloned leptospiral strain according to a standard protocol (International Committee on Systemic Bacteriology, 1984) and the homologous titre was determined by MAT.

5.1.6 Silver stain protocol

Solutions

Fixing solution: 10% acetic acid; 40% ethanol; 50% water (all water is deionised)

Sensitising solution: To 17g of sodium acetate, add 10ml of a 5% sodium thiosulphate solution, then 165ml water. Stir until dissolved then add 75ml ethanol

Silver stain solution: Dissolve 625mg of silver nitrate in 250ml water

Developer: Dissolve 6.25g sodium carbonate in 250ml water, then

add 100 µl formaldehyde (30%) just before use

Stopper: 3.65g EDTA in 250ml water

Method

1. Fix gel with 2 x 15 minute incubations with fixing solution. The gel is gently rocked at all times during this method.

2. Discard fixer and add the sensitising solution for 30 min.

3. 3 x 5 minute washes with water.

4. Replace with silver nitrate solution and incubate for 20 min.

5. 2 x 1 minute washes with water.

6. Replace water with developing reagent and monitor gel until the desired level of colour has developed (approx 5-15 minutes).

7. Remove developer and replace with stopper solution.

8. After 10-30 min in stopper, perform 3 x 5 min washes with water.

5.1.7 ELISA test procedure

Process	Details	Incubation
Antigen coating	Add 50µl coating antigen to each well Cover or wrap plate	Overnight at 4°C
Wash	Shake off antigen Add approximately 200µl wash buffer to each well and shake off Repeat wash x 6 Blot plate on absorbent material	
Block	Add 100µl blocking buffer to each well Cover or wrap plate	30 minutes at room temp.

Remove block	Shake off blocking buffer Blot plate on absorbent material	
Sample addition	Add 50µl dilution buffer to each of 8 wells in the first column Add 50µl of positive and negative controls to 2 adjacent wells each Add 50µl diluted sample to 2 adjacent wells each Cover or wrap plate	1 hour at room temp.
Wash	Shake off samples Add approximately 200µl wash buffer to each well and shake off Repeat wash x 6 Blot plate on absorbent material	
Conjugate addition	Add 50µl diluted conjugate to every well Cover or wrap plate	1 hour at room temp.
Wash	Shake off conjugate Add approximately 200µl wash buffer to each well and shake off Repeat wash *6 Blot plate on absorbent material	
Substrate addition	Add 50µl SureBlue substrate to every well	15 minutes at room temp.
Stop addition	Add 50µl H2SO4 (0.18mM) to each well	
Plate reading	Read the plate at 450nm	

APPENDIX 5.2

Extraction of leptospiral proteins using detergent lysis

Initial extraction of immunoreactive proteins from each leptospiral reference culture was attempted using detergent lysis, based on a method described by Biswas et al (2005). Leptospiral cultures identified as at least grade 4 (on a visual scale of 0-5 in terms of viability) were used – this corresponded to a density of approximately 2-4 x 10^8 leptospires/ml as determined by use of a bacterial counting chamber with dark-field microscopy. 2 ml of each of the 15 leptospiral culture was inactivated with 10 µl formalin and incubated at 37°C for 2 hours. Leptospiral death (lack of movement) was confirmed by dark-field microscopy. Each culture was centrifuged for 30 minutes at 4000G and 4°C and the pellets washed with 1ml 0.15M PBS three times, then centrifuged finally at 15000G. Pellets were resuspended in 50 µl of buffer containing? 4% Triton X (Sigma, Poole, Dorset), vortexed, and placed in a shaking incubator at 45°C for 4 hours. Each suspension was then centrifuged at 4000G for 30 minutes and the supernatant collected.

Supernatant protein content was determined using a bicinchoninic acid protein estimation assay (BCA: Perbio Science, Cramlington, UK) incorporating a bovine serum albumin (BSA) standard curve (Sigma-Aldrich, UK). Each sample was then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were mixed with an equal volume of SDS-PAGE reducing buffer and heated at 95° C for 10 minutes. 20 µl of each sample were run, along with molecular weight markers (Precision Plus Protein Unstained standards: Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) on a 12% SDS-PAGE gel with 4% stacking gel at 200V for 45 minutes. The gel was washed 3 times for 5 minutes in distilled water, stained with colloidal Coomassie G-250 (Imperial Protein Stain, Perbio Science) for 1 hour and de-stained in distilled water overnight. However, no distinct bands were visible other than one that corresponded to bovine serum albumin (BSA), which was present in the original leptospiral culture medium.

Detergent lysis was then attempted using much larger volumes of each culture.

100ml of each formalinised reference culture was centrifuged and washed as described above to obtain 15 pellets. The pellets were resuspended in 250 µl Triton X extraction buffer and placed in a shaking incubator at 200 rpm at 45C for 4 hours following the method from Biswas *et al* (2005). The solution was then centrifuged for 30 minutes at 15000g and 4C and the supernatant collected. Protein estimation was performed as above and the volume of each sample that contained 2 µg of protein was determined and mixed with an equal volume of SDS-PAGE buffer and heated at 95°C for 10 minutes. This volume was then subjected to SDS-PAGE at 200V for 45 minutes. Gels were stained with colloidal Coomassie G-250 as described above.

A few simple faint bands could be visualised, but at this point it was decided that urea extraction of leptospiral culture would be more likely to be successful. Urea is commonly used as a non-ionic chaotrope for protein solubilisation in protocols for the processing of cell lysates for two-dimensional electrophoresis (Cordwell, 2008; Rabilloud, 2009; Xavier et al., 2010) and ELISA antigen preparation (Maddison et al., 1982; Takimoto et al., 2008).

Extraction of leptospiral proteins using urea.

2ml of each formalinised culture was mixed together, divided into two Universal tubes and centrifuged for 30 minutes at 4000G and 4°C. The pellet was washed twice by resuspension in 20ml of PBS, vortexing and centrifugation for 30 minutes at 4000G and 4°C. 500µl of 8M urea extraction buffer (could put in appendix or under subheading at beginning of this section 10 millimoles Tris (buffer), 0.2% CHAPS (detergent) and 8M urea) containing Roche® EDTA-free complete mini protease inhibitors (Roche Diagnostics Ltd, Burgess Hill, UK) was added to each pellet, vortexed, and allowed to stand at room temperature (RT) for 15 minutes. The solutions were combined, transferred to an eppendorf and centrifuged for 30 minutes at 4000G and 4°C. The supernatant containing the solubilised proteins (urea soluble extract) was collected and the pellet containing insoluble debris retained.

100 µl of SDS-PAGE reducing buffer was added to the pellet, vortexed and

centrifuged for 30 minutes at 4000G and 4°C. The supernatant (SDS soluble extract) was run alongside the urea soluble extract (mixed 1:1 with SDS-PAGE reducing buffer) and molecular weight markers on a 12% SDS-PAGE Miniprotein gel with 4% stacking gel as before. 50 µl EMJH (the leptospiral culture medium) added to 50 µl SDS-PAGE buffer was used as a control. The gel was run at 100V for 90 minutes, to avoid over heating due to the presence of 8M urea in the samples and stained with colloidal Coomassie G-250 as described above. However, no significant bands were visible, indicating that protein had not been successfully extracted in detectable amounts.

Urea extraction was then attempted using larger volumes of leptospiral culture and a FastPrep® bead beater. 100ml of each formalinised reference culture was centrifuged for 30 minutes at 4000G and 4°C. The resultant pellets were washed 3 times in PBS and resuspended in 500 µl 8M urea extraction buffer containing Roche® complete mini EDTA-free protease inhibitors. Each sample was transferred to a FastPrep® tube and placed in a FastPrep® bead beater for 2 cycles of 40 seconds each, standing on ice for 5 minutes in between cycles before transfer into a clean eppendorf and centrifugation for 30 minutes at 15000G and 4°C. The supernatant was collected into a fresh eppendorf and ion exchanged into PBS using a Waters HiTrap® desalting column. A protein estimation assay was performed as previously described, and a volume representing 1 µg protein of each of the 15 cultures was subjected to SDS-PAGE at 100V for 90 minutes. The gel was stained with silver stain (See Appendix 5.1.6 for method) and distinct bands could be visualised indicating successful leptospiral protein extraction and separation.

APPENDIX 6

Reagents and buffers

MDCK cell growth medium

Eagle's Minimum Essential Medium (EMEM)

2mM glutamine

0.1mM non-essential amino acids

1.0mM sodium pyruvate

10% foetal bovine serum

100 U/mL penicillin

100 µg/mL streptomycin.

Urea extraction buffer (pH 7.5)

0.4% DTT

0.2% CHAPS

8M urea

20mM Tris-HCl

Alkaline-eosin buffer solution (pH 8.7):

120 mM NaCl

50 mM H_3BO_3

20 mM NaOH containing 0.1% sodium azide

0.4% Bovine serum albumin

0.05% Eosin.

SDS-PAGE reducing buffer

0.5M Tris HCl pH 6.8	5ml
Glycerol	4ml
10% sodium dodecyl sulphate (SDS)	8ml

2-Mercaptoethanol	2ml
0.1% Bromophenol blue	1ml

PAGE non-reducing buffer

0.5M Tris HCl pH 6.8

Glycerol

10% SDS

0.1% Bromophenol blue

SDS-PAGE gels:

12% separation gel:

Distilled water	3.35ml
1.5M Tris HCl pH 8.8	2.5ml
10% SDS	100µl
Acrylamide/Bis(30% stock)	4ml
10% ammonium persulfate	100µl
TEMED	10µl

4% stacking gel:

Distilled water	6.1ml
0.5M Tris HCl pH 6.8	2.5ml
10% SDS	100µl
Acylamide/Bis(30% stock)	1.3ml
10% ammonium persulfate	100µl
TEMED	10µl

Blocking buffer

TBS

Tween 80 (0.5%)

Bovine serum albumin (4%)